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Molecular Mechanisms Involved in Regulating Ion Transport in Colonic Epithelium

by

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Rebecca S. Lam

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Science is the search for truth – it is not a game in which one tries to beat his opponent, to do harm to others. We need to have the spirit of science in international affairs, to make the conduct of international affairs the effort to find the right solution, the just solution of international problems, not the effort by each nation to get the better of other nations, to do harm to them when it is possible.

No More War!
Linus Pauling, 1958

Dedicated to my family: Christopher, Nancy, and Joshua

ABSTRACT

The colon absorbs about one liter of fluid per day and its large transport capacity is attributed to the epithelium. Epithelial cells have the ability to absorb or secrete ions and water, but normally absorptive mechanisms predominate over secretory mechanisms. Colonic epithelial cells possess many ion channels and transporters working in concert to direct fluid movement. The balance of colonic fluid movement is tipped from absorption to secretion upon intestinal exposure to enterotoxins or inflammatory mediators. Alternatively, other pathophysiological conditions, such as the disease cystic fibrosis, can result in reduced secretion than normal. Many factors affect the behavior of epithelial ion channels and transporters, and ultimately colonic transport.

This thesis focuses on two mechanisms of regulating colonic epithelial ion transport: neurohormonal factors, via α-adrenergic receptors (absorptive) and via adenosine receptors (secretory), and cell membrane lipid composition (cholesterol and sphingomyelin). We used whole tissue transepithelial current measurements to study regulatory mechanisms of ion transport. Molecular techniques also elucidated the expression of certain potassium (K^+) channels. Basolateral K^+ channels are crucial for driving transepithelial anion secretion, and our studies emphasize their importance in colonic epithelial secretion.

Transepithelial ion transport studies showed that α_2-adrenergic G protein-coupled receptors inhibit colonic Cl^- secretion by inhibiting ATP-regulated K^+ (K_{ATP}) channels. This inhibition was mediated by $G_{i/o}$ proteins, but independent of Ca^{2+} or cAMP signaling.

The cell membrane houses ion channels and transporters in a complex lipid environment, and we explored the role of cell membrane lipids in regulating ion transport. Using lipid-altering agents, we found that cholesterol and sphingomyelin are important regulators of colonic secretion. Lipid rafts are membrane microdomains that are rich in cholesterol and sphingolipids, and we showed that the basolateral large conductance Ca^{2+}-regulated K^+ (BK) channels are regulated by lipid raft integrity.

We have also characterized cholesterol, independent of its role in lipid raft structure, as an important mediator of adenosine-stimulated epithelial secretion. The intermediate conductance Ca^{2+}-regulated basolateral K^+ (IK) channel was involved in adenosine-mediated secretion.

Our studies characterize novel regulatory mechanisms that are important for colonic epithelial transport. These results expand our knowledge of colonic function and may have significant therapeutic implications.

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ABBREVIATIONS

α_2AR	α_2-adrenergic receptor
AC	adenylyl cyclase
ACh	acetylcholine
ATP	adenosine triphosphate
BK	large conductance Ca^{2+}-sensitive K$^+$ channel
Ca^{2+}	calcium cation
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
Cl$^-$	chloride anion
ClC	voltage-gated Cl$^-$ channel
CNT	concentrative nucleoside transporter
ENaC	epithelial Na$^+$ channel
ENS	enteric nervous system
ENT	equilibrative nucleoside transporter
G protein	guanosine triphosphate-binding protein
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidylinositol
H$^+$	hydrogen cation
HCO$_3^-$	bicarbonate anion
IK	intermediate conductance Ca^{2+}-sensitive K$^+$ channel
I$_{sc}$	short-circuit current
K$^+$	potassium cation
K$_{ATP}$	ATP-dependent K$^+$ channels
KHS	Krebs-Henseleit solution
K$_{ir}$	inwardly rectifying K$^+$ channel
MβCD	methyl-β-cyclodextrin
MDCK	Madin-Darby Canine Kidney
mRNA	messenger RNA
Na$^+$	sodium cation
NBC	Na$^+$/HCO$_3^-$ cotransporter
NBD	nucleotide binding domain
NECA	5'-(N-Ethylcarboxamido)adenosine
NHE	Na$^+$/H$^+$ exchanger
NKCC	Na$^+$/K$^+$/2Cl$^-$ cotransporter
ORCC	outwardly rectifying Cl$^-$ channel
PDZ	PSD-95, discs large, ZO-1
PKA	protein kinase A (cAMP-dependent protein kinase)
SCFA	short-chain fatty acid
SK	small conductance Ca^{2+}-sensitive K$^+$ channel
SMase	sphingomyelinase
SUR	sulphonylurea receptor

CHAPTER 1

Introduction

1. *Epithelial Ion Transport*

1.1 Epithelial tissues

Epithelial cells function as barriers between the internal and external, or two different, environments. Surfaces of the body that are in contact with the external environment, such as the skin, airways, and intestine, are lined by epithelium consisting of a continuous layer of cells which can protect the inner tissues. In addition to acting as barriers, epithelial cells can secrete substances and transport molecules, such as ions. Ion transport across epithelia helps regulate cell volume, pH, and secretion. How epithelial cells regulate the specialized function of transepithelial ion transport is the main topic of this thesis.

Epithelial cells are polarized such that they have distinct apical and basolateral membranes. In the intestine, the apical membrane faces the lumen and the basolateral membrane faces the basement membrane, which acts to structurally support the epithelium, and blood vessels. The two membranes are morphologically and functionally different and many membrane proteins, including ion channels and transporters, are distributed in a polarized manner in epithelial cells. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) is found in the apical membrane of intestinal epithelial cells, whereas the Na^+/K^+-ATPase is found in the basolateral membrane. Protein trafficking initiates this distribution, and tight junctions maintain it. Lipid rafts (see Section 2.2.2) are one mechanism thought to be involved in the polarized trafficking of membrane proteins.

The two distinct membranes are maintained by the presence of tight junctions, which join two adjacent epithelial cell membranes together. The junctions are composed of a dense protein network of fibrils containing molecules of occludin (113) and claudin (112). Tight junctions give the epithelium its barrier function, but also act to prevent intermixing of the apical and basolateral membrane components (350).

Some epithelia are considered "tight" and others "leaky", corresponding to relatively low and high tight junction permeabilites, respectively. The presence of tight junctions confers a resistance across an epithelium; it is considered "leaky" if its transcellular resistance is more than its paracellular resistance. Colonic epithelium is considered "medium-tight" (122) and in mouse distal colon, transepithelial and paracellular resistances are on the order of 100 $\Omega\cdot cm^2$ and 3 $k\Omega\cdot cm^2$ (122), respectively. Transepithelial resistances vary along the length of the colon, with the proximal colon having lower resistances (58). Therefore, for substances to be transported across colonic epithelium efficiently, transcellular transport is vital.

In the space between the epithelial cells and subluminal to the tight junctions lie the paracellular, or lateral interstitial, spaces. When an epithelium secretes or absorbs electrolytes, water transport follows by osmosis. The contribution of the paracellular pathway in secretion and absorption is largely unknown. It is generally thought that movement through tight junctions occurs by simple diffusion, although there is evidence for tight junction permeability regulation in the intestine (18). Some disease states result in the epithelium becoming more leaky and paracellular water transport becomes more significant. For example, in the case of *Clostridium difficile* infection of the intestine, the epithelial barrier no longer functions properly due to the breakdown of tight junctions. This allows more water to flow into the lumen which aggravates the diarrhea (225).

Transepithelial water transport occurs through water channels, called aquaporins. Presently there are 11 identified isoforms of aquaporins named AQP0-10. In intestinal epithelium, AQP3 (307) and AQP4 (353) are localized to the basolateral membrane whereas AQP5 shows apical expression (219), and their presence clearly indicates a role for transepithelial fluid movement.

1.2 Colonic epithelium

The colon lies at the distal portion of the gastrointestinal tract, where it functions to store and eventually expel stool. The colon is also involved in the reabsorption of electrolytes and water, approximately 1.5 L per day in humans (71), and short-chain fatty acid (SCFA) uptake from luminal contents. Epithelial transport, therefore, is important in determining the absorptive or secretive capacity of the colon.

Colonic epithelium contains goblet cells and enteroendocrine cells (50). Epithelial cells comprise about 95% of the cells, and enteroendocrine cells make up the remaining 5%. Mucus covers the epithelial surface and is produced by the goblet cells (140). In addition to epithelial cells, the colon consists of several other cell types, including neural, vascular, muscular and immune cells.

The continuous cell layer of epithelium that makes up the colonic mucosa forms many invaginations called crypts. In mouse colon, there are about 700,000 crypts and 700 million epithelial cells (54). Crypts in mouse distal colon are approximately 160 μm in length and their openings are 10 μm in diameter (122). Figure 1•1 shows a histologic section of mouse colon mucosa. Two notable points from Figure 1•1 are: 1) that the crypts line up in parallel, with luminal openings shown by the arrow, and 2) the nuclei in

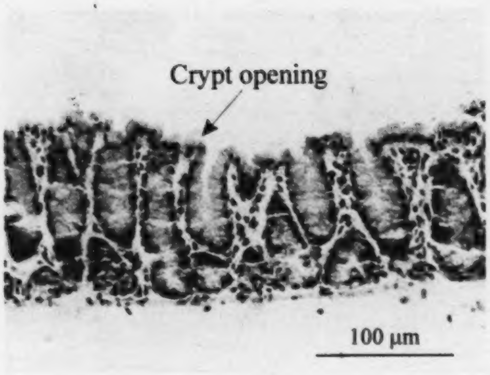

Figure 1·1 Mouse colonic epithelium
The tissue is oriented so the surface epithelium that faces the lumen is at the top of the picture. Tissue was dissected free of underlying smooth muscle, fixed in 4% paraformaldehyde, and embedded in Paraplast. Sectioning was done perpendicularly to the luminal surface at 8 μm thickness, and the tissue was stained with a nuclear stain (hematoxylin).

the epithelial cells lie close to the basolateral portion of the cells, which illustrates morphological polarity of the cells.

In addition to increasing the surface area of the colon, crypts are the source of new cells. Stem cells lie at the bottom of crypts and as the cells mature, they move up the crypt toward the surface where they are eventually sloughed off, or undergo apoptosis (108;167;249). Colonic epithelial cells have short life spans, lasting several days and the cell renewal rate in the intestine is among the highest in the body. In fact, as you are reading this paragraph, each crypt in your intestine has made one or two new cells. In mouse colon, a single crypt produces about 20 cells per hour (53), therefore, each crypt produces about 500 new cells per day.

Interest in the mechanisms responsible for intestinal secretion and absorption began in the early 1900s. Maurice Visscher published a 1938 paper showing that Cl^- and Na^+ were absorbed from the intestinal lumen. His results gave some insight into the process of intestinal absorption and secretion (160).

A model of colonic transport developed where absorptive and secretory processes were spatially separated and occurred in surface cells and crypt cells, respectively (361). Although generally correct, this idea was regarded as an oversimplification when isolated crypts were later shown to have absorptive properties (20;312) and surface epithelia were capable of secretion (170). Colonocyte properties change as they move from the crypt base to surface, and this will be exemplified in the next sections with respect to channel and transporter expression along the length of a crypt.

In general, the small intestine has been better studied than the colon. However, extrapolating data from small intestinal studies to colonic function is not correct. Large differences exist between these two areas of the intestine. For example, the colon does not participate in nutrient absorption from ingested food to the degree that the small intestine does, so apical glucose transporters - vital in small intestinal epithelium - are virtually absent in colon epithelium (215). However, a small fraction of colon epithelial cell energy is from glucose uptake on the basolateral membrane (247). The colon also possesses a unique resident bacterial population, which is largely uncharacterized. The colonic microflora digests leftover dietary fiber and starch into the SCFAs acetate, butyrate, and propionate, and colonic epithelial cells derive the majority of their energy from butyrate uptake (273). Because the colon has different functions, and therefore protein expression, from the small intestine, the review of secretion and absorption will largely focus on studies performed in colonic epithelial cells except in areas where there is a lack of colonic studies.

1.3 Colonic transport

Under normal conditions the colon functions as a net absorptive epithelia, but in response to secretagogues it can produce a net secretory response. Obviously the epithelium must have the capacity to both secrete and absorb, and a disruption in homeostasis can cause an excess of one or the other (examples of this are described in Section 3). In this context, absorption is defined as the movement of a substance from the apical to basolateral direction, and secretion is movement in the opposite direction. Net transepithelial ion transport is a result of absorptive and secretory processes mediated by the ion channels and transporters.

Transgenic mouse models lacking specific ion channels or transporters have been made, and they have given us greater insight into the role these channels and transporters play in intestinal transport. Several of these mouse models have been important for studying colonic transport, including mice that are deficient in the expression of CFTR, the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), Na^+/H^+ exchangers (NHE2 and NHE3), and H^+/K^+-ATPase, and where appropriate these models will be discussed further. Attention is also given, where available, to the spatial distribution of channels and transporters with respect to three areas: along the length of the colon (proximal and distal), along the crypt base-to-surface axis, and cell membrane localization (apical and basolateral).

A typical diet includes approximately 100 mmol Na^+ and Cl^- (280), and 70 mmol K^+ (285) per day. The kidney is vital for controlling the amount of ions secreted, however the colon also contributes to ion homeostasis. Under healthy conditions, Na^+ and Cl^- are reabsorbed from the lumen of the colon and K^+ is secreted so that excreted stool contains approximately 5 mmol Na^+, 2 mmol Cl^-, and 12 mmol K^+ (357). 90% of the water entering the colon is reabsorbed across the epithelium (71), and this process occurs due to osmotic drive from the electrochemical gradients formed by Na^+ and Cl^- reabsorption.

1.3.1 Colonic absorption

In health, there is a net absorption of Na^+ and Cl^-. Electroneutral absorption of Na^+ and Cl^- occurs through the apical Na^+/H^+ (NHE) and Cl^-/HCO_3^- (AE) exchangers. To date, the NHE family has nine members (229;239) of which three isoforms have been found in colonic epithelium. NHE1 is ubiquitously expressed and localized to the basolateral membrane of colonic epithelial cells (28;56), where it helps control intracellular pH and cell volume homeostasis. NHE2 and NHE3 are found in the apical membrane of colonic epithelial cells (28;29;56;154), and the contribution of each isoform to colonic absorption is not well known and controversial. NHE3-deficient mice showed greatly decreased levels of basal intestinal fluid (298) and Na^+ (119) absorption, and were observed to have diarrhea (298). In contrast, NHE2-deficient mice did not have decreased intestinal Na^+ absorption (119;297). The predominant isoform for basal Na^+ absorption is considered to be NHE3 (298), although other studies give evidence that it is NHE2 (49). Immunostaining (56) and mRNA (7) expression studies in murine colonic crypts show differential expression of NHE2 and NHE3, with NHE2 more abundant in crypt base cells and NHE3 predominantly in surface cells, whereas NHE1 is evenly distributed along the crypt length. Segmental differences also exist for NHE distribution along the length of the colon. This was shown in human colonic tissue for NHE2, which showed higher mRNA expression in the distal colon, whereas NHE1 and NHE3 were uniformly distributed from proximal to distal colon (89).

There is also evidence for a novel Cl^--dependent mechanism for Na^+/H^+ exchange (260) that acts independently of NHE2 or NHE3. In 2002 this exchanger, referred to as Cl-NHE, was cloned from rat colon (287), and it shows inhibition by the chloride channel blocker NPPB suggesting that Cl^- channels are responsible for the Cl^--dependence (261). Cl-NHE is postulated to be responsible for absorption in crypt cells (287).

Electrogenic Na^+ absorption was also shown to vary along the length of the colon, predominating in the distal colon (58) via Na^+ channels that are sensitive to amiloride (43;58). It is known that electrogenic Na^+ absorption occurs through apical epithelial Na^+ channels (ENaC), which shows higher expression in distal, compared to proximal, colonic epithelium (87). ENaC is a heteromer composed of three homologous subunits called α, β, and γ (44). ENaC also has a gradient distribution from colonic crypt base to surface and this supports the generalized absorptive role attributed to surface cells. Protein and mRNA expression are most abundant on the surface epithelial cells (87;268), so Na^+ absorption only appears in surface cells (171). Glucocorticoid and mineralocorticoid hormones are known to increase Na^+ absorption in the colon (19), and steroid-induced upregulation of colonic ENaC is due to activation of transcription of the β- and γ-subunits (268;327).

Na^+ absorption may also occur through novel pathways. The purinergic P2X receptors are ligand-gated non-selective cation channels that transport Na^+, K^+, and Ca^{2+} (347). P2X receptors have been found in human colon epithelial cells (337), where they may contribute to transepithelial absorption in response to ATP. In rat colon, there is evidence of non-selective cation channels in the apical membrane (107;253). After Na^+ enters the cell, it is extruded through the basolateral Na^+/K^+-ATPase, which completes the process of transepithelial Na^+ absorption.

Apical Cl^-/HCO_3^- exchangers mediate the electroneutral absorption of Cl^-. Studies in rat colon determined the presence of apical Cl^-/HCO_3^- exchange in colonic epithelium (256). There are three identified AEs named AE1, AE2, and AE3, which mediate the exchange of Cl^- and HCO_3^- across the cell membrane. An AE4 has also been cloned (344), but there is conflicting evidence about its transport activity and its function still needs to be defined. Apical Cl^-/HCO_3^- exchange is mediated by AE1 and the DRA (downregulated in colonic adenomas) protein (259). Interestingly, mutations in DRA cause congenital chloride diarrhea (150;226). As well as contributing to Cl^- absorption,

Cl^-/HCO_3^- exchangers are present in the basolateral membrane where they are involved in cell volume and intracellular pH regulation. AE1 and AE2 are found in the basolateral membrane of colonic epithelial cells (258;334), and in mouse colon epithelium AE2 showed greater expression in surface compared to crypt base cells (3).

Electrogenic absorption of Cl^- can occur through apical anion channels, namely CFTR, but the role of CFTR in intestinal Cl^- absorption is not well known. Absorbed Cl^- exits the cell through basolateral Cl^- channels. Cl^- channels shown to exist in the basolateral membrane of colon epithelium include outwardly rectifying Cl^- channels (ORCCs) in mouse (223), rat (77;78) and guinea pig (197), and the voltage-gated Cl^- channel CLC-2 in guinea pig (47;48). Relatively little has been characterized about the basolateral Cl^- channels in colonic epithelium, but their existence is unquestionable.

Active absorption of K^+ from the lumen is through apical H^+/K^+-ATPases in surface and crypt base cells (262), and K^+ exits via basolateral K^+ channels. H^+/K^+-ATPases belong to the P-type family of ATPases, which includes the Na^+/K^+-ATPase. The H^+/K^+-ATPases are heterodimers made up of α and β subunits (289). Two H^+/K^+-ATPases have been identified and can be characterized by their sensitivity to ouabain: one is ouabain-sensitive (72) and the other is ouabain-insensitive (289). The ouabain-sensitive H^+/K^+-ATPase is active in the apical membrane of surface and crypt base cells, whereas the ouabain-insensitive H^+/K^+-ATPase is active in the apical membrane surface cells only (262). Furthermore, H^+/K^+-ATPase activity is present in distal but not proximal regions of rat colon (72). Protein expression of a colonic H^+/K^+-ATPase α subunit (HKcα1) in the rat colon showed that this H^+/K^+-ATPase was restricted to the apical membrane of surface cells in the distal colon (186;288). This distribution suggests that HKcα1 encodes the ouabain-insensitive H^+/K^+-ATPase. Evidence for expression of the β subunit of the ouabain-insensitive H^+/K^+-ATPase is the protein called HKcβ, which coimmunoprecipitates with HKcα1 (286). In accordance with the important role of H^+/K^+-ATPases in K^+ absorption, HKcα1 knockout mice had a fecal K^+ excretion two times greater than wildtype mice (221). H^+/K^+-ATPase activity is also important for regulating intracellular pH.

1.3.2 Colonic secretion

Cl^- secretion through apical Cl^- channels is a major determinant for colonic fluid secretion. CFTR is the main apical Cl^- channel in colonic epithelium. As opposed to ENaC, CFTR mRNA expression is most abundant in the crypt base cells (330;342), where CFTR plays a large role in the secretory function of these cells. CFTR belongs to the ATP-binding cassette (ABC) family of proteins, which all have the ability to hydrolyze ATP. CFTR is a cAMP-sensitive anion channel residing in the apical membrane of intestinal epithelia. Phosphorylation by protein kinase A (PKA) activates CFTR and allows electrogenic anion efflux through the channel. Although commonly referred to as a Cl^- channel, it is more correct to call CFTR an anion channel since there is good evidence that HCO_3^- can also move through CFTR (250). In addition to its ion channel function, CFTR has been implicated in the regulation of many other processes including ENaC regulation (130;332), ORCC regulation (115), Cl-NHE regulation (261), mucin secretion (177), and aquaporin water transport (295). CFTR also contains PDZ domains, which allow it to interact with other proteins. A macromolecular signaling complex containing CFTR, the β_2-adrenergic receptor and ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50, also referred to as NHERF) was found in airway epithelial cells (230) and this represents one way for CFTR to both regulate cellular processes and to be regulated.

The vital role for CFTR-mediated secretion is apparent when considering the consequences of CFTR dysfunction on transepithelial ion transport. An example that will be discussed in Section 3.1 is the genetic disease cystic fibrosis (CF), where dysfunctional CFTR results in deficient epithelial secretion and dehydration of intestinal contents. As evidence of this, transgenic CF mice have a high morbidity due to intestinal obstruction (128).

It has been suggested that the high CF carrier rate in the general population is due to the "benefits" of CFTR dysfunction (254). This advantage would afford a resistance to bacterial-induced diarrheas, such as cholera, which cause Cl^- secretion through CFTR. Support for this idea comes from studies on heterozygote CF mice, which showed a decreased intestinal secretory response to cholera toxin (114).

A search for apical Cl^- channels that could take the place of CFTR is important because manipulating the activity of alternate Cl^- channels could be of therapeutic benefit to CF patients. In CF mouse small intestine a basal Cl^- secretion was present (136), suggesting that alternative apical Cl^- channels do exist. CaCCs (or CLCAs) are Ca^{2+}-sensitive Cl^- channels activated by Ca^{2+}/calmodulin protein kinase II (109). There is no clear evidence for CaCC expression in colon epithelial cells. Indeed, if CFTR is the most abundant apical anion channel in colon epithelium, this may be why CF causes severe gastrointestinal complications. Although there is one paper that published CaCC expression in the colon (131), it has been suggested that the detection was actually due to expression of closely related genes (110). However, CaCCs may take over the activity of CFTR in CF mouse lungs, which would explain the lack of pulmonary disease in CF mice (57;130).

Another class of Cl⁻ channels that could contribute to Cl⁻ secretion is the voltage-gated Cl⁻ channels (CLCs). The first CLC channel, ClC-0, was identified in the electric ray *Torpedo marmorata* (163) and there are now 9 different CLC genes identified in mammals: CLC 0-7, CLC-Ka, and CLC-Kb (162). Furthermore, the crystal structure for a bacterial CLC channel has been elucidated (92). CLC-1 (skeletal muscle localization), CLC-2 (ubiquitous expression), CLC-Ka (kidney, inner ear localization), and CLC-Kb (kidney, inner ear localization) are cell membrane channels, whereas the other CLCs are intracellular Cl⁻ channels (162). CLC-2 is expressed in human (199), guinea pig (47), and rat (199) colon epithelium. It was thought that CLC-2 might represent an alternative apical Cl⁻ channel, but several studies showed that CLC-2 had a basolateral localization. In the guinea pig colon, CLC-2 appears to localize on surface epithelium and not crypt base cells, and reside in the basolateral membrane (47;48), where it could contribute to Cl⁻ exit. Consistent with this function for basolateral CLC-2, mice that are both CFTR and CLC-2 deficient did not show exacerbated defects in colonic Cl⁻ secretion (369). However, the finding that CLC-2 localizes to tight junctions in mouse ileum (137) indicates that CLC-2 might have other functions, perhaps as a tight junction channel that could mediate paracellular Cl⁻ transport.

Basolateral K^+ channels are important for maintaining an electrical driving force for epithelial anion secretion (318). K^+ entering the cell through the Na^+/K^+-ATPase or NKCC is "recycled" out through the basolateral K^+ channels, which hyperpolarizes the basolateral membrane and helps maintain the processes of Na^+ absorption and Cl^- secretion. K^+ can also exit through apical K^+ channels, which mediate K^+ secretion into the lumen. Although it is known that apical K^+ channels in colonic epithelium contribute to K^+ secretion (299;362), their properties are less well characterized than the basolateral potassium channels. The presence of apical Ca^{2+}-regulated K^+ channels has been observed in rat colon (42;164;301).

K^+ channels make up a diverse and heterogenous group of channels. Structurally, they can be separated into two broad groups: those that have 2 transmembrane domains, and those that have 6 transmembrane domains. They are also classified by their nucleotide sequence homology, or according to their biophysical, pharmacological, or regulatory characteristics. These different ways to classify K^+ channels have made their nomenclature heterogenous and complex. In 1998 the bacterial KcsA channel was the first ion channel to have a resolved 3D structure from X-ray crystallography (84) and its structure confirmed the putative models of K^+ channels' pores.

Several types of basolateral K^+ channels commonly found in mammalian colon epithelial cells include: 1) cAMP-sensitive K^+ channels in rat (176) and human (206), 2) K_{ATP} channels in rat (67;157), and 3) Ca^{2+}-sensitive K^+ channels. The Ca^{2+}-sensitive K^+ channels are grouped according to their single channel conductances: small (SK), intermediate (IK) and big (BK). Ca^{2+}-sensitive K^+ channels expressed in colonic epithelium include: 1) SK channels in rat (22;37;164;358) and human (206), 2) IK channels in rat (111;164) and human (76;284), and 3) BK channels in rabbit (345) and rat (37;164). It is likely that other types of K^+ channels exist in colonic epithelial cells and are only waiting to be discovered. Recently the voltage-gated K^+ channel Kv1.3, thought to be found only in nerves and lymphocytes, was shown to be expressed in rat colonic epithelium (133). Many of the basolateral K^+ channel studies have used human, rabbit, rat, and guinea pig colon epithelium, and there is less literature using mouse colon epithelium. However, identified basolateral K^+ channels in mouse colon epithelial cells include a cAMP-sensitive K^+ channel (91;211;212) consisting of KCNQ1/KCNE3 multimers (210;296) and an intermediate conductance Ca^{2+}-sensitive K^+ channel (212) made up of KCNN4 subunits (91;212;210).

Another vital player in colonic secretion is NKCC, which is located on the basolateral membrane of colonic epithelial cells (65) where it is a pathway for Cl^- entry and a requirement for Cl^- secretion to occur. It is an electroneutral cotransporter with a stoichiometry of $1Na^+:1K^+:2Cl^-$ (138). There are two major isoforms of NKCC: NKCC1, whose expression has been shown in human (65) and mouse (9;129) colonic epithelium, and NKCC2, whose expression was examined in mice and shown to be restricted to the kidney (159). Unlike CF mice, NKCC1-deficient mice do not have intestinal obstruction and have a relatively mild intestinal pathology, showing dilated crypts (9;129). This suggests that there are alternative pathways for basolateral Cl^- entry that contribute to the maintenance of Cl^- secretion.

Na^+/HCO_3^- cotransporters (NBCs) are another pathway for anion transport across the basolateral membrane. Evidence for NBC colonic expression has been found in human (2), rabbit (302), rat (41) and mouse (8;302). Since the initial cloning of a human (40) and an amphibian (276) renal NBC in 1997, many members of this family have been cloned and there are now four NBC isoforms called NBC1-4 (321). In fact the NBCs belong to the broader solute carrier (SLC) 4 family of HCO_3^- transporters that includes the anion exchangers AE1-4. NBC1 is an electrogenic transporter and transports $1Na^+:3HCO_3^-$ in the kidney epithelium (275;322). However, in most other tissues it appears to have a stoichiometry of $1Na^+:2HCO_3^-$ (275;321). Although it is well established that NBCs contribute to colonic basolateral HCO_3^- entry (257) and NBC1 is expressed in the colon (302;303), further characterization of NBCs in this location awaits.

The Na^+/K^+-ATPase is ubiquitously expressed and it has the crucial function of maintaining the Na^+ and K^+ gradients across the cell membrane. It resides in the basolateral membrane of epithelial cells (169), where it also contributes to colonic secretory function. For every molecule of ATP hydrolyzed, the Na^+/K^+-ATPase transports three Na^+ ions out of the cell and two K^+ ions into the cell (169). The Na^+/K^+-ATPase maintains an inwardly directed Na^+ gradient that drives secondary transport, such as that through NKCC1. Evidence that Na^+/K^+-ATPase activity contributes to Cl^- secretion is that ouabain, an inhibitor of the Na^+/K^+-ATPase, blocks Cl^- secretion (16).

The transepithelial transport of an ion does not occur in isolation, but instead affects, and is affected by, the movement of other ions. For Cl^- secretion to occur there will be an opening of Cl^- channels in the apical membrane, an influx of ions through NKCC, and K^+ exit from the cell. It is well established that K^+ exit via basolateral K^+ channels hyperpolarizes the cell and will drive Cl^- secretion (178). In fact, basolateral K^+ channels are necessary for Cl^- secretion to occur since basolateral K^+ channel inhibitors block Cl^- secretion (205). The integrated response of a colonic epithelial cell's ion channels and transporters will determine its secretory response. Figure 1•2 and 1•3 show the channels and transporters involved in colonic epithelial secretion and absorption, with attention to crypt axis distribution.

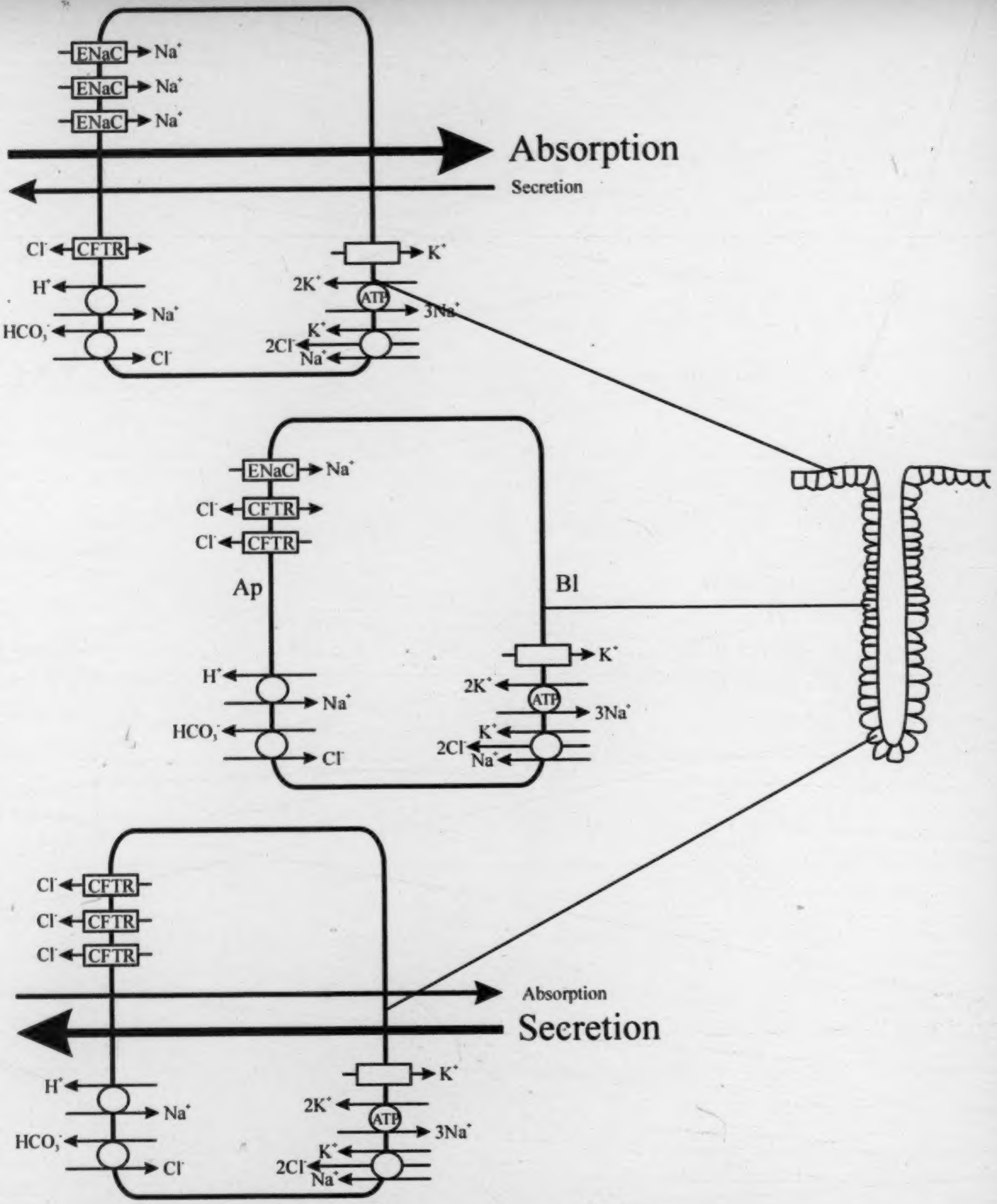
ENaC
Na+
ENaC
Na+
ENaC
Na+
Absorption
Secretion
Cl-
CFTR
K+
H+
2K+
ATP
Na+
3Na+
HCO3-
K+
2Cl-
Cl-
Na+
ENaC
Na+
Cl-
CFTR
Cl-
CFTR
Ap
Bl
K+
H+
2K+
ATP
Na+
3Na+
HCO3-
K+
2Cl-
Na+
Cl-
Cl-
CFTR
Cl-
CFTR
Cl-
CFTR
Absorption
Secretion
K+
H+
2K+
ATP
Na+
3Na+
HCO3-
K+
2Cl-
Na+
Cl-

Figure 1·2 Transepithelial ion transport in distal colonic epithelium
The expression of ion channels and transporters along the crypt base-to-surface axis in the distal colon. Electrogenic Na^+ absorption via ENaC is most prominent in the surface epithelial cells. Electroneutral NaCl absorption occurs throughout the crypt length. CFTR expression is greater in the crypt base cells. Ap = apical membrane, Bl = basolateral membrane

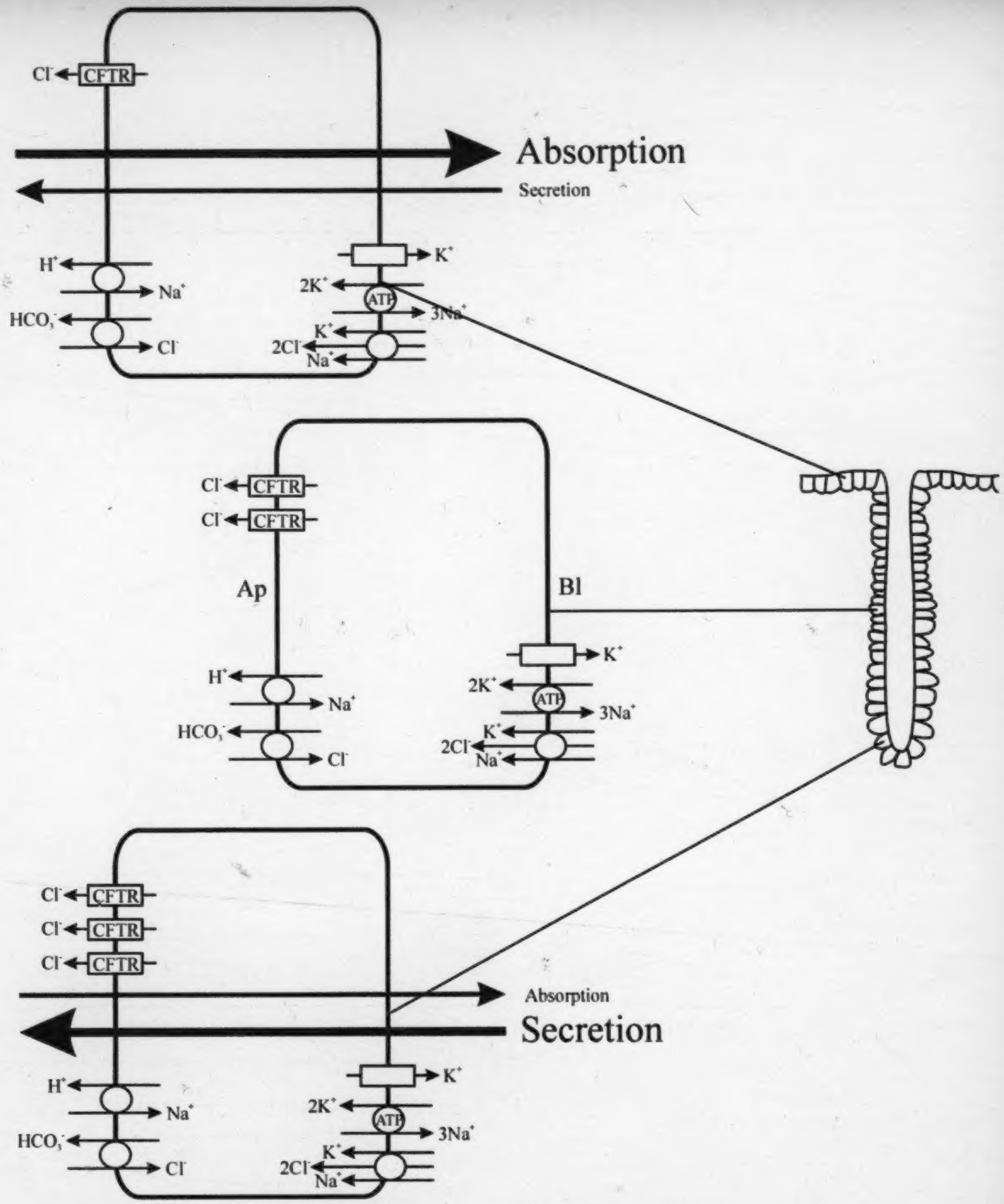
Cl⁻ CFTR
Absorption
Secretion
H⁺
Na⁺
HCO₃⁻
Cl⁻
K⁺
2K⁺
ATP
3Na⁺
K⁺
2Cl⁻
Na⁺
Cl⁻ CFTR
Cl⁻ CFTR
Ap
Bl
H⁺
Na⁺
HCO₃⁻
Cl⁻
K⁺
2K⁺
ATP
3Na⁺
K⁺
2Cl⁻
Na⁺
Cl⁻ CFTR
Cl⁻ CFTR
Cl⁻ CFTR
Absorption
Secretion
H⁺
Na⁺
HCO₃⁻
Cl⁻
K⁺
2K⁺
ATP
3Na⁺
K⁺
2Cl⁻
Na⁺

Figure 1·3 Transepithelial ion transport in proximal colonic epithelium
The expression of ion channels and transporters along the crypt base-to-surface axis in the proximal colon. Electroneutral NaCl absorption occurs throughout the crypt length, and CFTR expression is greater in the crypt base cells. Ap = apical membrane, Bl = basolateral membrane

One of the main methods to study transepithelial transport is to use the short-circuit current (I_{sc}) technique. Hans Ussing pioneered this technique in the 1950s and his first paper using this technique, along with $^{24}Na^+$, showed that the I_{sc} measured across frog skin epithelium was due to sodium transport (346). Any epithelial tissue that forms resistant monolayers of cells can be mounted in Ussing chambers, where it is voltage-clamped at zero and then transepithelial current is recorded, which corresponds to ion movement across the tissue. For Ussing chamber experiments, colonic epithelial tissue is dissected free of the underlying smooth muscle layers (Figure 1•1) and typical I_{sc} traces are shown in Figure 1•4.

2. *Physiological Mechanisms Regulating Ion Transport*

2.1 Neurohormonal mechanisms

The activation or inhibition of transepithelial ion transport is under the regulation of excitatory and inhibitory influences. Regulatory factors include neurotransmitters and hormones that are released from endocrine, nerve, immune, or epithelial cells. Neurohumoral signals exert their effects through many intracellular signaling messengers and pathways. Cyclic AMP, cGMP, and Ca^{2+} are well-studied messengers that have all been shown to affect ion transport, and therefore regulate colonic function. Although largely neglected, the presence of immune cells and their mediators in the gastrointestinal tract also contributes to secretory or absorptive functions of the colon epithelium. In fact, the gastrointestinal tract holds the majority of immune cells in the body. Physiologically speaking, an integration of neural, immunological, humoral, and epithelial influences will all contribute to colonic epithelial ion transport. This section will

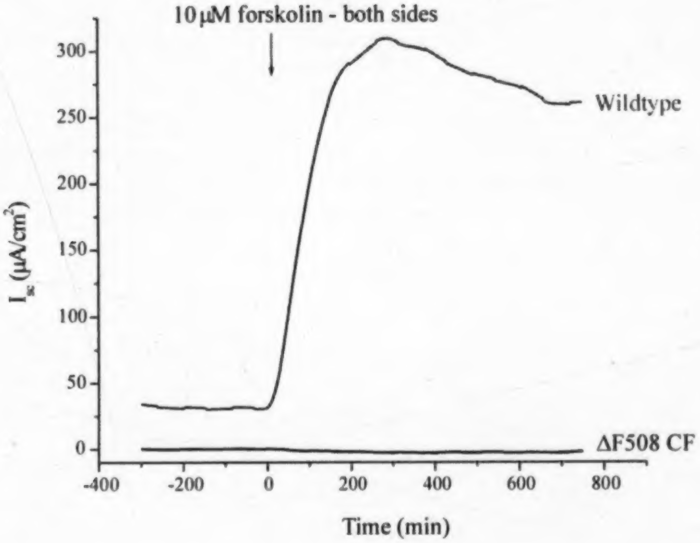

Figure 1·4 Short-circuit current (I_{sc}) recordings comparing responses between wildtype and ΔF508 CF mice
The response to forskolin (AC activator, 10 µM bilateral) in wildtype mice or a transgenic mouse model of CF. CF mice show a lack of cAMP-mediated Cl^- secretion as well as lower basal I_{sc}.

present two main mechanisms that regulate ion transport, and specific studies looking at these two regulatory mechanisms will be detailed in subsequent chapters.

2.1.1 Neural innervation

The colon is extensively innervated by the extrinsic fibers of the autonomic nervous system (ANS) and the intrinsic nerves of the enteric nervous system (ENS) to coordinate healthy gut function. Autonomic innervation of the colon comes from the parasympathetic vagus and splanchnic nerves and the sympathetic nerves from the superior mesenteric, inferior mesenteric, and pelvic ganglia (80). The ENS possesses intrinsic activity and consists of Auerbach's myenteric plexus and Meissner's submucosal plexus. The two plexuses are made up of many ganglia interconnected by nerve fibers. Myenteric plexus nerves have cell bodies residing between the circular and longitudinal muscle, and submucosal plexus nerve cell bodies reside between the circular muscle and the mucosa. In general, the myenteric plexus oversees the contractile function of the circular and longitudinal smooth muscle, and therefore the mixing and propulsion of the intestinal contents. The submucosal plexus mainly controls chloride secretion by crypt cells and blood flow to the intestine. Parasympathetic nerves synapse with nerves in the myenteric plexus, which also communicate with nerves of submucosal plexus (168), and the two plexuses are coordinated and communicate with each other and the ANS to control secretion, motility, and blood flow. The ENS's intrinsic activity is apparent when severing the sympathetic and parasympathetic fibers to the intestine, since digestive functions are not compromised (6).

The intestine is extensively innervated and neurotransmitter release can act on non-neural cells. This is evident by the presence of receptors for classical neurotransmitters on non-neural cells. Colon epithelial cells express both adrenergic and cholinergic receptors. Stimulation of α_2 adrenergic receptors (α_2ARs) on the basolateral membrane (348) of colonic epithelia is known to inhibit secretion (151;300). There are 3 subtypes of α_2 adrenergic receptors called α_{2A}, α_{2B}, and α_{2C}. Colonic epithelium contains the α_{2A} subtype (348), and the receptor shows a higher expression in the proximal colon and in the crypt base cells (305;348). Catecholamines are released from the sympathetic nerves innervating the mucosa and postganglionic nerves primarily secrete noradrenaline. There are also 4 types of β adrenergic receptors (β_1-β_4), and the β_2 subtype is found in human colon epithelial cells (271). Rat colonic epithelium shows functional expression of β_3 adrenergic receptors also, and a β_3 agonist caused an increase in secretion (300).

Muscarinic receptors are also present in colonic epithelia where, in response to acetylcholine (ACh), they stimulate secretion (161). ACh can come from several sources, including parasympathetic neurons and efferent neurons from the submucosal plexus (61;161). There are 5 subtypes of muscarinic receptors (M_1-M_5) and it is the M_3 subtype that is found in the basolateral membrane of colon epithelial cells (198). It is well established that stimulation of M_3 receptors activates Ca^{2+} to induce colonic secretion (198;251). It has been suggested that mechanical stimulation from the presence of luminal feces will activate intestinal neurons and, through submucosal reflexes, a basal level of low chloride secretion for maintaining lubrication (62).

2.1.2 Adenosine

The secretory nature of the epithelium can also be altered in response to hormones and other molecules. Colonic epithelial cells express receptors for many secretagogues including adenosine. Adenosine is a purine nucleoside that is well known to have effects on intestinal epithelial secretion. Both colonic cell lines (14;31) and colonic tissue (125) show increased secretion in response to adenosine. Adenosine may come from several sources where it acts on colonic epithelium to mediate secretion.

Local production of adenosine by the colonic epithelial cells occurs due to the action of the ecto-5'-nucleotidase, also called CD73 (328). This enzyme converts 5'-AMP into adenosine, which can then act on its receptors, or be transported into the cell via nucleoside transporters. In the small intestine there is evidence for luminal adenosine production from degradation of microbial nucleic acids (291) and endogenous nucleic acids from the high turnover rate of intestinal epithelial cell shedding (244). During inflammatory states, the concentration of adenosine can greatly increase due to adenosine production from inflammatory cells. Intestinal inflammation often results in disturbed secretory responses. Intestinal mast cells secrete adenosine (13), and neutrophils (213) and eosinophils (269) release 5'-AMP. 5'-AMP can be converted into adenosine by CD73, which can then act on colonic epithelial cells and stimulate secretion. Large amounts of ATP may be produced in the gut lumen from the killing of microbial invaders, such as the enteropathogenic *Escherichia coli.* ATP metabolism will yield adenosine, which contributes to the colonic secretory diarrhea (63). Adenosine can also act in a paracrine or autocrine manner. Local actions of adenosine were evident when hypoxic conditions promoted endogenous adenosine release in colon epithelial cells, and this stimulated Cl^- secretion (220). Therefore, under hypoxic conditions adenosine might play a role in converting the net absorptive function of colonic epithelium into net secretion.

Adenosine acts through receptors belonging to the purinergic receptor family. There are four subtypes of adenosine receptors called A_1, A_{2A}, A_{2B}, and A_3. They comprise the P_1-purinergic receptors, which are G protein-coupled receptors (GPCRs). Each receptor subtype has its own classical signaling pathway characterized based on the G protein that it is coupled with. A_1 couples with $G_{i/o}$, A_{2A} with G_s, A_{2B} with G_s or G_q, and A_3 with G_i or G_q (263). It is well known that adenosine activates Cl^- secretion in colon epithelial cells (14;31;125). A_{2B} receptors are present in T84 cells and human colon tissue (329). In T84 cells, activation of A_{2B} receptors results in increased cAMP production and increased anion secretion (329). Adenosine receptors are present in both the apical and basolateral membranes of colonic epithelial cells (15).

Epithelial cells scavenge extracellular adenosine through nucleoside transporters. This keeps local adenosine concentrations relatively low, and this corresponds to the low level of basal Cl^- secretion. There are two major classes of nucleoside transporters, the concentrative (or Na^+-dependent) nucleoside transporters (CNTs) and the equilibrative (or Na^+-independent) nucleoside transporters (ENTs). CNTs can transport nucleosides against their concentration gradient, whereas ENTs transport nucleosides along their concentration gradients. Presently there are three identified subtypes of CNTs, named CNT1-3 (127), and four ENTs, named ENT1-4 (10). ENT1 and ENT2 are distinguished by their sensitivity to the drug nitrobenzylthioinosine (NBTI or NBMPR) with ENT1 being NBTI-sensitive and ENT2 being NBTI-insensitive. Interestingly, ENT3 has an intracellular localization and is relatively insensitive to NBTI (11). ENT4 function is not yet well defined. There is functional evidence of ENT expression in T84 cells (227) and ENT1 and ENT2 mRNA is expressed in T84 as well as Caco-2 cells (355). There are conflicting results looking at CNT expression in colonic epithelial cells. In a study looking at mRNA expression in T84 and Caco-2 cells, CNT1 and CNT2 expression was not found (355). Additionally, CNT activity was not found in rabbit colon (272). CNT1 is expressed in the brush border, but not basolateral, membrane of the rat small intestine (141), so if it is present in colon epithelium it may have an apical localization. Mouse colonic tissue showed the presence of mRNA for ENT1-3, and low levels of CNT2 and CNT3 (207).

Adenosine homeostasis is the result of the activity of several enzymes. The actions of two enzymes result in very low intracellular concentrations of adenosine. Intracellular adenosine kinase creates AMP from adenosine, whereas adenosine deaminase metabolizes adenosine to inosine. Since the ubiquitous enzyme adenosine deaminase has rapid actions, adenosine likely acts in a more paracrine/autocrine manner under basal conditions than as a circulatory hormone. In fact it appears that high (up to mM) concentrations of exogenous adenosine are required to stimulate Cl^- secretion in colonic tissue (125).

2.2 The role of cell membrane composition

Membrane proteins reside in the lipid bilayer, and the contribution of the effect of lipids on membrane protein activity is not well understood. There is a general understanding that membrane proteins require the proper lipid environment in order to function normally, but knowledge about the specific regulation of these proteins by lipid constituents is lacking. Cholesterol is a major lipid in cell membranes and it has been linked with regulating many membrane effects. It is undoubtedly an important molecule for regulating some membrane proteins, but its role in colonic ion transport has not been thoroughly studied. Cholesterol is also a defining part of lipid rafts, which are microdomains in the cell membrane that have regulatory roles. Therefore, cholesterol's regulatory properties may be due to its association with these specific structures in the cell membrane.

2.2.1 Cholesterol

The distribution of total cell cholesterol is such that the majority resides in the cell membrane. Cell membranes are enriched in cholesterol, whereas intracellular membranes, including the endoplasmic reticulum (ER) and mitochondrial membranes, contain much less (60). In fibroblasts, up to 90% of total cellular cholesterol resides in the cell membrane (182), although cell membrane cholesterol content appears to vary depending on the cell type and anywhere from 65-90% of total cholesterol is reported in different cell membranes (180;181;356).

It is not understood why levels of cell membrane cholesterol are relatively high compared to intracellular membranes. However, several molecules are important for cholesterol transport and expression in the cell membrane. The high sphingomyelin (SM) content of the cell membrane maintains the cholesterol content (359). Similar to cholesterol, the majority of total cell SM is located in the cell membrane (180;182;315) and cholesterol favours interactions with SM more than with other phospholipids

(235;248;359). SM degradation alters the cell membrane cholesterol distribution by mobilizing cholesterol for esterification and causing a decrease in cholesterol biosynthesis (52;135;314;315). On the other hand, cholesterol levels also affect the homeostasis of SM (190). The association between cholesterol and sphingomyelin is the basis for the formation of lipid rafts (Section 2.2.2).

Caveolin, a protein found in caveolae (see section 2.2.2), may be important for cholesterol transport and cell surface expression. Caveolin is a cholesterol-binding protein (228), and newly synthesized cholesterol appears in caveolae first and then non-caveolae cell membrane (102;316). Caveolin mRNA levels are also linked to cellular cholesterol levels (21;101).

Cholesterol has four fused rings, referred to as A, B, C, and D. The rings are arranged in a planar way, and a 3β-hydroxyl group gives cholesterol its hydrophilic property so that the molecule is amphipathic. The planar molecule orients with its polar hydroxyl group facing the aqueous intracellular and extracellular environments and the hydrophobic rings lie parallel to the hydrocarbon chains of the phospholipids. The structure and orientation of cholesterol give the cell membrane rigidity by reducing the freedom of movement of phospholipid hydrocarbon chains. The fluidity of the cell membrane describes the motional freedom of molecules in the lipid bilayer and there is abundant evidence that cholesterol decreases cell membrane fluidity (25;45;54;121;123).

The main sources of cholesterol in intestinal epithelial cells are from: 1) extracellular uptake from plasma lipoproteins, 2) uptake from the lumen of the intestine, or 3) biosynthesis. Each of these processes will sequentially be described here.

Basolateral extracellular uptake of cholesterol is achieved through receptor-mediated uptake of plasma lipoproteins. There are five classes of lipoproteins, classified according to their density: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL). LDL and HDL carry the majority of plasma cholesterol. The apolipoproteins, part of the lipoproteins, interact with cell membrane receptors or enzymes and so determine the receptor-mediated uptake of cholesterol into the cell. After internalization, the lipoproteins are degraded and the cell can then use the released cholesterol.

Cholesterol is transported in LDL as cholesteryl esters, with the hydroxyl group esterified. Cellular uptake of LDL is receptor-mediated, and as would be expected for plasma cholesterol uptake, LDL receptors are present in the basolateral membrane of colonic epithelial cells (104). Once inside the cell, LDL is delivered to lysosomes where the cholesteryl esters are hydrolyzed to release free cholesterol for cell use. HDL mediates the reverse process of cholesterol efflux, which is essential since cholesterol is not degraded and must be sent to the liver for conversion to bile acids. This process also involves apolipoprotein interaction with the cell membrane, and in fact lipid rafts may mediate this interaction (118).

ATP-binding cassette transporter A1 (ABCA1) is an integral part of cholesterol efflux. Like CFTR, ABCA1 belongs to the ABC family of transporters. ABCA1 is found in the basolateral membrane of colonic epithelial cells (234) where it mediates the transport of cholesterol across the cell membrane (267). A rare autosomal recessive disease of reverse cholesterol transport is Tangier disease. Characteristics of this disease include very low plasma HDL levels and accumulation of cholesterol esters in macrophages throughout the body, including the tonsils, liver, spleen, and intestine (106). Interestingly, Tangier disease is due to mutations in ABCA1 (279).

Although there is advanced understanding about the biosynthetic pathway for cholesterol production, little is understood about the molecular processes involved in dietary intestinal cholesterol absorption. It is generally accepted that the small intestine absorbs bile acid micelles containing cholesterol through the apical membrane. It was previously assumed that cholesterol absorption from luminal contents was passive (132), however there is good evidence now for protein-mediated cholesterol uptake (147;174;341). In fact inhibitors of intestinal cholesterol absorption (2-azetidinones such as ezetimibe/Zeria® and sterol glycosides) are emerging as novel pharmacological treatment for high plasma cholesterol levels (39;75). Since so little is understood about intestinal cholesterol uptake though, the action of these drugs is unknown. It was recently shown that Niemann-Pick C1 Like 1 (NPC1L1) protein, which shares ~50% homology to NPC1 (69), is expressed in the gastrointestinal tract and is important for cholesterol absorption in the intestine (4). NPC1L1-null mice were not responsive to ezetimibe suggesting this is the pathway for 2-azetidinone actions (4). NPC1L1 is found in the colon (although its expression is low compared to the small intestine) suggesting that the colon has a small capacity for luminal cholesterol absorption (4). However, two potential cholesterol-binding proteins in the apical membranes of intestinal epithelial cells were not found in colon epithelial cells (174), suggesting that luminal cholesterol absorption does not occur in the colon.

Intestinal epithelial cells possess the necessary machinery to synthesize cholesterol from one molecule of acetyl coenzyme A. In a pathway involving over 20 separate reactions, one of the reactions is the formation of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). This reaction is catalyzed by the enzyme HMG-CoA reductase, and is a major regulatory step in cholesterol biosynthesis. The statin class of drugs are HMG-CoA reductase inhibitors used for the treatment of hypercholesterolemia.

Cholesterol is synthesized in the ER and then transported to other membranes. Cell membrane cholesterol is not static, but is continually travelling to and from the ER, where cholesterol is synthesized. Total cell membrane cholesterol moves to the ER and back to the cell membrane with an estimated half-time of 40 min (179). Within minutes, small changes in cell membrane cholesterol provoke large responses in ER cholesterol (183), and the cholesterol content of a cell is under tight regulation.

Through the pioneering work of Joseph Goldstein and Michael Brown, the main proteins involved in cellular cholesterol homeostasis are known. Cells maintain their cholesterol content through a transcriptional regulatory system. The genes regulating cholesterol synthesis have promoters containing sterol regulatory element (SRE) sequences. Sterol regulatory element-binding proteins (SREBPs) are a group of transcription factors that reside in the ER membrane. There are three identified SREBPs named SREBP-1a, SREBP-1c, and SREBP-2 (156;306;368). The N-terminal domain of SREBPs contains transcription factor activity (243), and the C-terminal domain is a regulatory domain that interacts with SREBP-cleavage-activating protein, or SCAP (283). When the cellular cholesterol level is too low, transport of the SCAP-SREBP complex from the ER to the Golgi occurs. This allows the N-terminal domain of SREBPs to be cleaved off by two sequential cleavages (282) and then to travel to the nucleus. Two Golgi proteases carry out the cleavages: Site-1 protease (S1P) first cleaves the SREBP into two halves (90), and Site-2 protease (S2P) cleaves the N-terminal fragment that will move to the nucleus and bind the SRE (282). SCAP is required for S1P cleavage (155), and there is evidence that cholesterol directly binds SCAP (255). This would allow cholesterol to suppress proteolysis by S1P, and downstream cholesterol synthetic events. So SCAP has at least two functions, binding to SREBP and escorting it to the Golgi, and sensing the cholesterol levels. Binding of the SREBP N-terminal domain to the SRE activates the transcription of enzymes for cholesterol synthesis such as HMG-CoA reductase (156;317), farnesyl diphosphate synthase (95), and squalene synthase (134), which alleviates the initial low cholesterol signal.

One molecule identified to be involved in intracellular cholesterol transport is the Niemann-Pick C1 (NPC1) protein (46). The importance of such proteins is apparent because abnormalities in intracellular cholesterol transport lead to diseases, such as Niemann-Pick type C (NPC) disease. Fibroblasts from people with NPC have normal LDL uptake, but LDL-derived cholesterol accumulates in lysosomes. Interestingly, NPC1 contains the cholesterol-sensing domain that is found in SCAP (68), suggesting it may function in targeting cholesterol-containing vesicles. The precise biological function of NPC1 remains unknown.

How does cell membrane cholesterol affect membrane protein function or transepithelial ion transport? The answer is not known, but there is plenty of evidence showing that membrane cholesterol affects membrane protein activity. Modulating membrane cholesterol content has been shown to have effects on the function of receptors (24;121;232) and ion channels (191;202;274), as well as membrane proteins in intestinal epithelial cells (34;88). Another way that cholesterol may affect membrane proteins is through its association with lipid rafts.

2.2.2 Lipid rafts/caveolae

Singer and Nicholson's Fluid Mosaic Model (311) described the idea that the cell membrane contains "proteins floating in a sea of phospholipids" in 1972. This model has been expanded to support the concept that proteins are distributed non-randomly in the cell membrane. In the 1990s, a mechanism for membrane protein organization was proposed based on the observation that some epithelial proteins exhibited insolubility in nonionic detergents (36;290). The concept of lipid rafts was first described by Simons and Ikonen in 1997 (308), and since that time over 1500 papers have been published in this area. Lipid rafts are cell membrane microdomains that are rich in cholesterol, glycosphingolipids (GSLs), and certain membrane and membrane-associated proteins. Phospholipids usually have unsaturated acyl chains and form "liquid-disordered" phases, whereas GSLs have longer saturated acyl chains (cholesterol preferentially partitions between these saturated chains) that will pack more tightly in the lipid bilayer to form "liquid-ordered" phases. These liquid-ordered domains are also known as lipid rafts and one could imagine them floating among the more disordered phospholipids.

Like cholesterol, GSLs are also enriched in lipid rafts. Compared to other membrane lipids, GSLs have a relatively low abundance in the cell membrane. Ceramide is at the core of the structure of all GSLs. GM1 ganglioside is a common resident GSL in rafts, and it is often used as a lipid raft marker.

Rafts are believed to play a key role in several important processes in epithelial cells, including cellular signaling and lipid and protein trafficking (309;310). By inclusion or exclusion of certain proteins, rafts can act as membrane protein regulators. The pathogenesis of certain diseases may depend on lipid rafts. Rafts or raft proteins have been shown to be targets in bacterial infections such as *Clostridium difficile* (233), *Escherichia coli* (304), *Mycobacteria tuberculosis* (117), *Pseudomonas aeruginosa* (126), *Vibrio cholerae* (371), and *Salmonella typhimurium* (116), and viral infections such as Ebola (94), Human Immunodeficiency Virus (236), and measles (216).

Biochemically, raft proteins are identified by their isolation in cold nonionic detergents, like Triton X-100, where they are present in detergent-resistant membranes/fractions (DRMs/DRFs), also known as detergent-insoluble glycolipid-enriched complexes (DIGs). Some proteins appear to be permanent residents in lipid rafts; examples include glycosylphosphatidylinositol (GPI)-anchored proteins like 5'-nucleotidase (328), placental alkaline phosphatase (36), hemagglutinin (313), and Thy-1 (105).

Interestingly, many types of ion channels have been found in detergent-insoluble fractions, including transient receptor potential channels (204), pacemaker channels (12), P/Q-type Ca^{2+} channels (336), Kir3.1 (74), Kv1.3 (23), Kv1.4 (365), Kv1.5 (218), and Kv2.1 (217;274) channels. In epithelial cells alone, lipid raft studies have shown ion channel association with rafts. ENaC was found in lipid rafts in kidney epithelial (A6) cells (148). An olfactory cyclic nucleotide-gated channel, CNGA2, was found associated with lipid rafts in olfactory epithelium (33). In MDCK cells, lipid rafts appear to target large conductance Ca^{2+}-sensitive K^+ (BK) channels to the apical membrane (35). Although there are no studies that specifically show the association of colonic epithelial ion channels with lipid rafts, NHE3 was found associated with apical lipid rafts in the small intestine (196). Lipid rafts may indeed play a role in regulating colonic epithelial ion transport.

Some proteins are dynamically associated with rafts. The adenosine A_1 receptor, the epidermal growth factor receptor, and P-glycoprotein translocate out of rafts upon stimulation (120;184;224). On the other hand, proteins that do not normally reside in rafts may become raft-associated upon stimulation (120). CFTR is not found in lipid rafts under basal conditions (32), but evidence suggests that it may become raft-associated due to *Pseudomonas aeruginosa* infection (173). What we think of as a lipid raft may only represent a snapshot in time of a dynamic cell membrane. Subsets of rafts may associate and disassociate continually so that a cell membrane is in a constant state of flux.

With respect to the polarized nature of epithelia, rafts present one system that epithelial cells can employ to maintain their distinct apical and basolateral membranes. Initially, the function of rafts was largely studied in Madin-Darby canine kidney (MDCK) epithelial cells, and rafts were proposed to deliver certain newly synthesized proteins from the *trans*-Golgi network (TGN) to the apical cell membrane (99;175;200). For example, GPI-anchored proteins are localized to the apical membrane of epithelial cells (200), and rafts can aid the apical delivery of GPI-anchored proteins. There is good evidence that this is the case, since raft disruption perturbs the sorting of apical raft proteins (165;277). Caveolin-1 has also been shown to play a role in apical transport of influenza virus hemagglutinin (HA) in epithelial cells (292). There are no simple rules for the polarized sorting of proteins in epithelial cells, and different cell types can exhibit different sorting mechanisms. Apical proteins in MDCK cells are sorted through the TGN, but in retinal pigment epithelial cells HA appears to take a more convoluted route, showing up in the basolateral membrane and then transcytosing to the apical membrane (27). In small intestinal epithelial cells it was shown that apical membrane rafts contribute to the sorting of the apical enzyme aminopeptidase N (144).

Many signaling molecules are associated with lipid rafts and so rafts may act as centres for signal transduction. For example, inhibition of volume-regulated anion channels (VRACs) by the c-Src tyrosine kinase requires c-Src association with lipid rafts (343). Both transient receptor potential-canonical channels TRPC1 and TRPC3 appear to assemble in caveolar multimolecular signaling complexes (203;204). Whole signaling complexes have been defined and their residence in lipid rafts makes sense, since having all the molecules in close proximity would create efficient and rapid signal transduction as well as minimize cross-talk. Other proteins may also be associated and help to form these molecular complexes. "Scaffolding proteins", like SAP-97, have been shown to associate with caveolin-3 and also to regulate Kv1.5 activity (103).

Excellent data that supports lipid raft structure *in vivo* has looked at the lateral movement of single molecules in the cell membrane. Both raft lipids (79) and proteins (252) show less lateral diffusion than non-raft markers. The actin cytoskeleton may restrain the lateral mobility of rafts and the proteins in them. A model has been proposed where cytoskeleton "corrals" confine cell membrane protein mobility (188). Cytoskeletal or cytoskeletal-associated proteins have been found in lipid raft proteomes. Proteomic analysis of monocyte lipid rafts showed the presence of actin, actinin, and vimentin (195). Similarly, raft proteomics in other cell types shows the association of cytoskeletal proteins with rafts (324;352). Ion channels and transporters have been shown to associate with cytoskeletal components. For example, the BK channel interacts with the microtubule-associated protein 1A (242). It is reasonable to assume that the cytoskeleton represents a way for some proteins to reside in rafts.

One technical approach used to study rafts has been to manipulate cholesterol or sphingolipid levels pharmacologically, thereby disrupting raft stability. Cyclodextrins (121;274) and filipin (121) can acutely affect membrane cholesterol and disrupt lipid rafts, whereas the statin family of drugs can be applied to cells for long-term suppression of cholesterol biosynthesis (144). Application of cyclodextrins to cells has been shown to affect the activity of several types of ion channels including volume-regulated anion channels (191), hyperpolarization-activated cyclic nucleotide-gated (HCN4) channels (12), an α subunit of the olfactory cyclic nucleotide-gated (CNGA2) channel (33), and Kir2.1 channels (274). Membrane sphingolipid levels can also be altered by drugs that inhibit biosynthesis, such as fumonisin B_1 (185), or by using sphingomyelinase to deplete sphingomyelin (121). Treating cells with polyunsaturated fatty acids (PUFAs) in culture was shown to alter lipid composition and protein localization to rafts (331;360). *In vivo*, methods of disrupting lipid rafts have also been employed. Mice were fed a diet enriched in *n*-3 PUFAs for two weeks after which colonocytes (209) and T-cells (96) showed significantly lower levels of cholesterol and sphingomyelin in their raft fractions.

There is evidence that there are subsets of lipid rafts distinguished by certain resident proteins (124;214). Caveolae are considered one subtype of lipid raft. Caveolae were originally recognized morphologically as flask-shaped invaginations of about 70 nm in diameter in the cell membrane of endothelial (240) and epithelial cells (366). They contain resident proteins called caveolins, of which there are three isoforms named caveolin-1, caveolin-2, and caveolin-3. Caveolins are cholesterol-binding proteins that have a bent-hairpin structure. They are able to polymerize (228) and it is believed that this is how the cell membrane bends for invaginations to form. Caveolin-1 and caveolin-2 are coexpressed in many cell types (293;294), whereas caveolin-3 is localized to muscle cells (335). Mice have been made that lack each of the caveolins, and the mice fared better than they were expected to. Caveolin-1-deficient mice lack caveolae and caveolin-2 expression is also decreased (85;265), confirming the coexpression of the two proteins. As well, the mice exhibit lung and vascular pathophysiology. Mice deficient in caveolin-2 have caveolae, but these mice also have pulmonary dysfunction, suggesting an important role for caveolin-2 in the lungs (266). Caveolae are anchored to the actin cytoskeleton through caveolin-1 associating with the protein filamin (325;340), and this may help to maintain the shape of caveolae.

Although caveolar structure is known, little is understood about the function of caveolae. As with lipid rafts in general, caveolae have been implicated in a number of important cellular processes, and this was the main reason that the viability of caveolin-deficient mice was so surprising. Caveolae may be involved in endocytosis, like the clathrin-coated pits, however they show great stability at the cell membrane that would rule out their participation in constitutive endocytosis (340). Caveolae also may be important in cellular signaling. For example, endothelial cell nitric oxide signaling depends on caveolins and caveolae (85;265). There are several lines of evidence suggesting that caveolins are tumor suppressor genes (172) and are downregulated in cancers (17;187). However, there is also evidence for upregulation of caveolin-1 in some cancers (194;367). Furthermore, caveolin-1-deficient mice do not have a greater incidence of tumor formation (265).

Caveolae are present in the basolateral, but not the apical membrane (292;351), of epithelial cells. There is conflicting evidence for the expression of caveolins in colon epithelial cells. Studies in Caco-2 cells have shown caveolin expression and localization to the apical membrane (100), whereas others show a lack of endogenous caveolin expression (192;351) and upon gene transfection with caveolin-1, caveolae form in the basolateral membrane (351). HT-29 cells express caveolin-1 and caveolin-2 (38). In one study using human colonic tissue, caveolin-1 expression was shown (17), however in another study caveolin-1 (5;192) and caveolin-2 (5) were not expressed in human colonic epithelium, but tissue samples from patients with ulcerative colitis showed caveolin-2 expression in the apical membranes of colon epithelial cells (5). Caveolae isolated from mouse and rat colon epithelial cells show the expression of caveolin-1 (209). The presence of caveolae in colon epithelial cells would suggest their role in signaling and membrane protein regulation in these cells, and perhaps caveolae are important in colonic epithelial ion transport.

3. *Pathology of Ion Transport Mechanisms*

There are dire consequences when the equilibrium between intestinal secretion and absorption is disturbed. A fine balance from the contribution of the epithelial ion channels and transporters, as well as the intracellular and extracellular factors that regulate them, maintains healthy intestinal function. This section presents two examples of altered CFTR function on opposite ends of the transport spectrum: deficient secretion and excess secretion.

3.1 Hyposecretion: cystic fibrosis

Cystic fibrosis (CF) is an inherited (autosomal recessive) disease that afflicts 1:2,000-3,000 of the Caucasian population (97). The disease is due to mutations in the CFTR gene, first cloned in 1989 (270), that results in a dysfunctional anion channel. Like other members of the ABC family of proteins, CFTR structure contains 2 nucleotide-binding domains (NBD1 and NBD2) where ATP can bind (270). CFTR also has a regulatory R-domain which contains sites that can be phosphorylated by PKA (55) and PKC (51). The C-terminal PSD-95/DLG/ZO-1 (PDZ)-binding domain (354) allows CFTR to associate with several other proteins. Binding of PDZ domain proteins to CFTR mediates cytoskeletal interactions, regulated signaling, and the inclusion of CFTR in macromolecular complexes. Although more than 1000 mutations in the CFTR gene have been identified (see www.genet.sickkids.on.ca/cftr/), the most common mutation, accounting for ~ 70% of CF cases (208), is a 3 base pair deletion resulting in a loss of phenylalanine at position 508 (ΔF508) in NBD1 (270). ΔF508 results in improper intracellular processing of CFTR so that the protein is retained in the ER and then prematurely degraded. Other mutations may affect CFTR production, regulation, or conduction, but ultimately the result of all mutations is a lack of Cl^- transport.

The most severe complication of CF is lung disease. With a lack of Cl^- secretion in the airways, there is also a deficiency in fluid secretion and thick mucus develops that contributes to lung disorder. Mucociliary clearance of this thick mucus is impaired, and there is also lung inflammation. CF patients are more susceptible to lung infections from *Pseudomonas aeruginosa* (281), and lung function progressively declines with age. Because CFTR resides in many epithelia, CF patients typically have other complications, including exocrine pancreatic insufficiency (139), infertility (81) and intestinal dysfunction.

Defective Cl^- transport can have significant consequences for intestinal function, and 85-90% of CF patients experience some form of gastrointestinal complication (241). About 15% of newborns with CF have meconium ileus, where the distal ileum and proximal colon are dilated due to obstruction with thickened pellets of meconium (201). In adult CF patients, ileal obstruction with fecal material is known as meconium ileus equivalent or distal intestinal obstructive syndrome, and occurs in about 15% of the CF population (86;278). Maldigestion often occurs due to pancreatic insufficiency, and stools appear "greasy" with visible fat droplets. A high caloric diet with supplementary vitamins and pancreatic enzyme replacement helps with dietary deficiencies and digestion of fats and proteins. Other intestinal problems in CF include bloating, excess gas, constipation (278), and intussusception (153).

Eleven transgenic mouse models of CF have been generated and described in the literature so far, including three ΔF508 CF mouse models (59;349;370). Interestingly, mouse morbidity is due to intestinal dysfunction, whereas lung function is decent. It is thought that other apical Cl^- channels compensate for the lack of functional CFTR in the lungs of CF mice (57;130). CF mice show gastrointestinal obstruction that is similar to human complications, namely blockage or even rupture in the distal small intestine and colon (320) due to luminal contents that are more viscous than normal. Histopathology of CF mouse intestine shows mucus accumulation in crypts as well as goblet cell hyperplasia (93). In one study looking at histological features of ΔF508 CF mouse tissue, the colon showed thick secretions, but no change in goblet cells numbers (339). The severe intestinal complications result in a higher rate of morbidity for CF mice (264;320;370), but it has been shown that using liquid diets can increase their life span (93;166). Presumably this diet is easier on intestinal function. Figure 1•4 shows the colonic epithelial response to the AC activator forskolin in ΔF508 CF vs. wildtype mice. There is a complete lack of cAMP-stimulated chloride secretion in the ΔF508 CF mice. Also evident is the lack of basal Cl^- secretion, since baseline I_{sc} is lower in CF mice. CF mice are a useful experimental model for learning more about CF mechanisms and physiology, especially with respect to the intestine, because of the similarities between CF mouse and human gut function.

The CF genotype may affect intestinal phenotype. The $Cftr^{tm1Hgu}$ model of CF mouse has a higher survival rate and shows a cAMP-mediated Cl^- secretory response ~50% of that for wildtype mice (82;319). The production of ~10% of normal CFTR may be the reason these mice have a greater survival rate (83). As well, the $Cftr^{TgHm1G551D}$ mouse shows some cAMP-mediated Cl^- secretion (73), and the equivalent mutation in human CF presents a lower rate of meconium ileus compared to ΔF508 patients (142). In fact, ΔF508 CFTR can function as a Cl^- channel (193), and so one therapeutic strategy is to improve the secretory response of ΔF508 CFTR. Support of this strategy was shown when adding PKA type II activators together with phosphodiesterase class I and III inhibitors to ΔF508 CF mouse intestine activated a discernable secretory response (326).

It is postulated that CFTR downregulates ENaC activity. This would aggravate the Cl^- secretory defect because it would cause epithelia to not only under-secrete, but also to over-absorb. Evidence for this hypothesis is that Na^+ transport in CF airway epithelia was shown to be upregulated (30). Human CF colonic tissue has been shown to have an increased response to the ENaC inhibitor amiloride (238). On the other hand, some studies have not shown a difference in amiloride-sensitive Na^+ absorption in colonic tissue from CF patients (145).

The intestinal complications in CF arise as result of a variety of factors. At the epithelial cell level, improper functioning of CFTR alters the secretory function and CF intestinal epithelium is characterized by a lack of cAMP-stimulated secretion.

3.2 Hypersecretion: secretory diarrhea and cholera

Certain stimuli, like bacterial enterotoxins, can trigger a large volume of fluid secretion into the lumen of the gut, resulting in diarrhea. This is an excellent simple defense mechanism to flush out the microbe or noxious substance. The basic sequence of events is that toxin-receptor interactions occur at the apical membrane of epithelial cells, and this activates intracellular signaling processes that lead to an increase in transepithelial Cl^- secretion. Diarrhea is not often life-threatening in the first world, but poverty, war, and overcrowding can lead to contaminated water supplies and contribute to the fatal consequences of dehydration in developing countries.

Cholera is a well-studied stimulus of secretory diarrhea, and a good example of how alterations in cellular signaling ultimately affect transepithelial ion transport. A 1959 study showed that adding filtrate from *Vibrio cholerae* cultures into rabbit intestine resulted in fluid secretion (70), proving that a bacterial-released toxin caused the diarrhea. After release of the toxin from the bacteria, the toxin binds to intestinal epithelial cells to initiate its actions. The enterotoxin produced by *V. cholerae* is called cholera toxin, which is a protein consisting of an A subunit and five B subunits. The A and B subunits both share over 80% homology with the *Escherichia coli* enterotoxin A and B subunits (66;323), which also induce diarrhea. The B subunits bind to the GM1 ganglioside (152;222), hence cholera's dependence on lipid rafts for infectivity. Cholera toxin has been found to associate with lipid rafts in T84 cells, and binding to rafts was essential for toxin activation of secretion (364). After binding to GM1, the A subunit is endocytosed and enters a retrograde trafficking pathway via early endosomes (333) to the TGN and then to the ER. Internalization and transport of cholera toxin has been shown to be raft- and cholesterol-dependent in colon epithelial cells when sequestration of cholesterol inhibited toxin transport to the TGN (237;363). Once inside the ER, the A subunit undergoes proteolytic cleavage to release the A1 domain that is responsible for the toxin's enzymatic activity. *V. cholerae* secretes a protease that can mediate this cleavage (158), but it has been suggested that the cleavage occurs due to the action of endogenous epithelial proteases, such as protease activity within the apical early endosomes in T84 cells (189).

It is thought that the A1 fragment translocates from the ER into the cytosol through the Sec61p channel (146). Sec61p channel is normally used for transporting newly synthesized proteins from the cytosol into the ER, but it also transports misfolded proteins for proteasomal degradation in the cytosol (143). Once in the cytosol, the A1 fragment imposes its enzymatic activity on G_s proteins. It irreversibly modifies the G_s α

subunit so that it over-stimulates AC and this causes increased levels of cAMP, which affect cAMP-mediated events including the activation of PKA. With respect to secretory actions, PKA phosphorylates CFTR to stimulate intestinal secretion. There is an established role for CFTR in secretory diarrhea and evidence for this comes from studies that show CFTR inhibitors can block cholera-induced secretion in T84 cells, and mouse and human intestine (338). It follows that CFTR inhibitors may be good therapeutic tools for treating cholera.

Elevated levels of cAMP also indirectly affect secretion. For example, platelet activating factor is activated which induces the synthesis of phospholipase A_2 (PLA_2)-activating protein, and this then results in PLA_2 activation (246). PLA_2 then stimulates arachidonic acid release which results in prostaglandin (PG) production, including PGE1 and PGE2, which also activates the secretory response (245). There are other secretagogues that are brought into action to propagate the response to cholera, including 5-hydroxytryptamine (231).

V. cholerae produces other toxins in addition to CT. One is called zona occludens toxin (ZOT) and like its name suggests, it affects tight junctions by increasing their permeability (98). Recent findings also suggest that the B subunits are not merely passive receptor-binding molecules, but they may also activate intracellular signaling molecules like extracellular signal-regulated kinases (26).

It has been suggested that the high CF carrier rate in the general population is due to the "benefits" of CFTR dysfunction (254). This advantage would afford a resistance to bacterial-induced diarrhea, such as cholera, which cause Cl^- secretion through CFTR. Support for this idea comes from studies on heterozygote CF mice, which showed a decreased intestinal secretory response to cholera toxin (114). However, there are studies performed in mice (64) and humans (149) that refute this hypothesis.

The net result of cholera toxin exposure is a large loss of ions and fluid into the gut lumen. This secretory diarrhea is due to overactivation of CFTR and the accompanying water loss is severe. Cholera results in diarrhea containing as much as 20 L of fluid per day (1), as well as an increased output of fecal electrolytes.

4. Thesis objectives

Epithelial ion transport determines the secretory nature of the colon and many mechanisms regulate the transepithelial movement of ions. The aim of the studies for this thesis is to characterize certain mechanisms regulating colonic epithelial ion transport. The main goals are:

1. to further characterize the established neurohumoral mechanisms of α_2-adrenergic-mediated absorption and adenosine-mediated secretion in colonic epithelium;
2. to determine the influence of cell membrane composition on colonic epithelial secretory control.

The findings reported in this thesis contribute to our understanding of mechanisms regulating colonic epithelial ion transport. These studies provide important insights into colonic epithelial ion transport mechanisms in health and disease states.

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CHAPTER 2

Regulation of Cl^- secretion by α_2-adrenergic receptors in mouse colonic epithelium

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INTRODUCTION

The α_2-adrenergic receptors (α_2ARs) belong to the seven transmembrane domain superfamily of G protein-coupled receptors (GPCRs), and mediate many of the physiological effects of the native catecholamines, adrenaline and noradrenaline (17). The primary signal transduction pathway of α_2ARs is through pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins, which leads to inhibition of adenylyl cyclase and a reduction in intracellular cAMP (6). However, under certain circumstances α_2ARs can also couple to G_s proteins leading to activation of adenylyl cyclase (11). Other physiological signaling pathways mediated by α_2ARs include stimulation of phospholipases (A_2, C and D), and inhibition of voltage-gated Ca^{2+} channels (15). Three distinct α_2AR subtypes have been described (α_{2A}, α_{2B}, and α_{2C}AR) based on molecular and pharmacological criteria (17). These subtypes exhibit different cellular and tissue distributions, suggesting distinct physiological functions.

The presence of α_2ARs in the intestinal mucosa has been demonstrated in earlier studies (36). These receptors were shown to be of the α_{2A} subtype, and their distribution suggested preferential localization in the basolateral membranes of the proximal colon. The source of native α_2AR agonists may be the noradrenergic fibers that extensively innervate the intestinal mucosa, endocrine cells within the epithelial layer, or circulating catecholamines. While the presence of α_2ARs on enterocyte membranes implies a direct interaction between catecholamines and the epithelium, the mechanisms of α_2AR-mediated effects and the nature of their molecular interaction with ion channels remain poorly defined.

Classically, regulation of epithelial transport processes occurs in response to agents that alter cyclic nucleotide or $[Ca^{2+}]_i$ levels, affecting mainly apical anion channels and basolateral K^+ channels. Although CFTR Cl^- channels represent a major pathway for anion movement across the apical membrane, the contribution of outwardly rectifying Cl^- channels, Ca^{2+}-dependent Cl^- channels and members of the ClC Cl^- channel family may also be important (2;34). At least four biophysically and pharmacologically distinct types of K^+ channels have been shown to contribute to the basolateral K^+ conductance: a cAMP-activated K^+ channel (KCNQ1), an intermediate conductance Ca^{2+}-activated K^+ channel (IK-1), a large conductance Ca^{2+}-activated K^+ channel (BK), and ATP-dependent K^+ channels (K_{ATP}) (8;27;31). These channels are thought to play a crucial role in the regulation of the overall process of chloride secretion.

α_2AR agonists have been shown to inhibit electrolyte secretion in human colonic epithelial cell lines (20;40), rabbit ileum (12) and rat jejunum (38). Although this type of regulation may be of clinical and pharmacological relevance in diseases characterized by abnormal intestinal secretion, the molecular mechanisms involved in this process are not well understood. Therefore, our main objective was to identify ion channels and transporters affected by α_2AR agonists. Our data indicate that the main targets of α_2AR action are basolateral K_{ATP} channels. These channels are inhibited by a process that requires activation of $G_{i/o}$ proteins but is independent of the cAMP- or Ca^{2+}-mediated pathways.

MATERIALS AND METHODS

Epithelial cells

The colonic epithelia used in this study came from four dfferent strains of mice: BALB/c, NMRI, C57BL/6J and cystic fibrosis (CF) mice. The breeding colony of CF mice (B6.129S6-*Cftr*^{tm1Kth}, Jackson Laboratory, Bar Harbor, ME) was housed in a specific pathogen-free environment (Health Sciences Laboratory Animal Services, University of Alberta). Breeder pairs were heterozygous for a CTT deletion mutation of the CFTR gene, which results in the loss of a phenylalanine residue in exon 10 and corresponds to the human position 508. Pups were weaned at 21 days of age, and genotyped by PCR amplification of ear clip genomic DNA according to established methods (Jackson Laboratory, Bar Harbor, ME). Pups were either wild-type homozygous (+/+), heterozygous (+/ΔF), or homozygous for mutant CFTR (ΔF/ΔF) (*Cftr*^{tm1Kth}, referred to as CF). No differences in the behavior of intestinal epithelium from CFTR(+/+) and CFTR(+/ΔF) mice have been found (42), and no distinction between the two types is made in this study. All experiments described in this study were carried out with the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta.

Mice were killed by CO_2 narcosis, and 6 cm long pieces of colon were removed from ~2 cm below the caecum and immediately placed in cold Krebs-Henseleit (KH) solution containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. The colons were opened up, and the muscle layers were dissected away. Usually, two pieces of 0.2 cm^2 were taken from proximal and distal colon, and mounted in Ussing chambers. In experiments requiring Cl^--free KH solution, NaCl and KCl were replaced by equimolar sodium gluconate and potassium gluconate, respectively, and 2.5 mM $CaCl_2$ was replaced by 5 mM calcium gluconate to compensate for the Ca^{2+}-buffering capacity of gluconate. In experiments requiring HCO_3^--free solution it contained (mM): 141 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 10 HEPES, pH 7.4.

Transepithelial measurements

Standard techniques were used in Ussing chamber studies. The tissues were bathed on apical and basolateral sides with 10 ml of KH solution, which was warmed to 37°C and continually circulated with a gas lift by bubbling with 95% O_2-5% CO_2. Chemicals were added from concentrated stock solutions and both chambers were continuously and separately perfused to ensure proper oxygenation and stirring of the solutions. The transepithelial potential difference was clamped to zero using a DVC 1000 amplifier (WPI, Sarasota, Fl) and the resulting short-circuit current (I_{sc}) was recorded through Ag-AgCl electrodes and 3 M KCl agarose bridges. The I_{sc} was allowed to stabilize for 10-15 min before application of α_2 agonists or other tested chemicals. Positive currents were defined as anion secretion or movement from the basolateral to the apical side. The transepithelial resistance was continuously monitored and calculated, using Ohm's law, by measuring current changes in response to 0.5 mV pulses. The data were collected and stored using a PowerLab 8SP series (ADInstruments, NSW 2154, Australia).

For basolateral membrane K^+ current measurements apical NaCl was replaced by equimolar potassium gluconate, while basolateral NaCl was substituted with sodium gluconate and the Ca^{2+} concentration was increased to 5 mM in both solutions. In addition, 100 μM ouabain was added to the basolateral compartment to inhibit the Na^+/K^+-ATPase. Subsequent permeabilization of the apical membrane with nystatin (90 μg/ml) allowed measurement of K^+ current as these ions move down their concentration gradient through basolateral K^+ channels.

Unidirectional Ion Fluxes

To measure chloride flux from the basolateral to apical side, epithelia were short-circuited as described above. Three μCi of $^{36}Cl^-$ (Amersham Pharmacia Biotech, UK) was added to the basolateral side, 30 min allowed for equilibration and then two 0.5 ml samples were taken from the apical side and replaced with fresh KH solution; this point was considered time zero. Samples were taken thereafter at 10-min intervals for the next 30 min, followed by the addition of UK 14,304 and further sampling for another 30 min. Basolateral samples taken just prior to UK 14,304 addition gave the specific activity for chloride. This information together with that from the apical samples allowed calculation of the Cl^- flux change caused by UK 14,304. This change was compared to the change in the I_{sc} response, calculated from the integral of the I_{sc} versus time record. $^{36}Cl^-$ fluxes in the apical to basolateral direction were measured in exactly the same fashion, except that the radioisotope was added to the apical bathing solution.

Measurement of intracellular Ca^{2+} concentration (performed by Ms. Fenglian Xu)

Changes in $[Ca^{2+}]_i$ were measured in isolated colonic crypts using fura-2 as the reporter molecule. A ~6 cm long piece of mouse colon was everted, tied at both ends and filled with low calcium (LC) solution as previously described (10). The LC solution contained the following (mM): 127 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES, 5 EDTA, bovine serum albumin (BSA) 1%, pH 7.4. The distended and everted colon was submersed in LC solution at 37°C for 5 min with gentle shaking. At the end of 5 min the colonic preparation was shaken vigorously for 2 min, the tissue discarded and the crypt suspension separated by gentle centrifugation. The crypts were resuspended in KH solution and loaded with 2.5 μM fura-2 AM at 37°C for 15 min, and then washed with KH solution to remove untrapped dye before the beginning of each experiment. Groups of cells (typically 5) were illuminated with light delivered from an HBO 100 W mercury lamp via a ×40, 1.3 numerical aperture, oil-immersion objective. Cell fluorescence was analyzed with an imaging system (TILL Photonics, Eugene, OR), which included a monochrometer capable of switching the excitation wavelength in <1 ms and a cooled CCD camera synchronized to integrate the emitted light at each excitation wavelength. Ratiometric imaging of fura-2 fluorescence was performed at 0.2 Hz with sequential 340/380-nm excitation. Each fluorescence image was collected at 640 × 480 pixels resolution via a long-pass 510 nM dichroic mirror/emission filter and integrated for 10 ms. After subtraction of background fluorescence at each wavelength of excitation, the fluorescence ratio (R) of fura-2 at 340-nm excitation/380-nm excitation, was displayed as a continuous record showing the time course of changes of R from an individual region of interest.

Conversion of R into $[Ca^{2+}]_i$ was performed in separate calibration experiments, in which individual cells were dialysed in whole cell recording with intracellular solutions of known $[Ca^{2+}]$ and fura-2 (0.1 mM, potassium salt from Molecular Probes). The three solutions for this calibration were identical to those previously used for calibrating indo-1

(35) and had $[Ca^{2+}]$ of <0.1 nM, 212 nM, and 15 μM, respectively. R values were converted to $[Ca^{2+}]_i$ using the following equation (16)

$$[Ca^{2+}]_i = K(R - R_{\min})/(R_{\max} - R)$$

In this study, the value of R_{min} was 0.132, R_{max} was 3.4, and K was 2,723.6 nM.

Cyclic AMP radioimmunoassay (performed by Dr. Anthony Ho)

A radioimmunoassay procedure was used in which the samples were acetylated before analysis (19). Colonic epithelia (20 mm^2) prepared as for I_{sc} recording, were exposed either to 1 μM UK 14,304, or to 10 μM forskolin for 10 min in 50 μl of KH solution, either with or without IBMX (100 μM) at 37°C. Acetic anhydride, 50 μl (final concentration 5 mM) was added to stop the reaction. The whole tissue was then frozen and thawed three times, to disrupt the cells, using solid CO_2. Aliquots were taken for protein estimation while the remainder was boiled for 5 min, centrifuged at 20,000 g and used for immunoassay. Each measurement was the average of triplicate determinations.

Tissue noradrenaline measurement (performed by Maria A. Vieira-Coelha)

The noradrenaline assay was performed by HPLC with electrochemical detection as previously described (37). Briefly, 0.5 ml tissue samples in perchloric acid were placed in 5 ml vials with 50 mg alumina, and the pH was adjusted to 8.6 with Tris. The adsorbed catecholamines were eluted from the alumina with 200 μl of 0.2 M perchloric acid on Costar Spin-X microfilters, and 50 μl of the eluate was injected into HPLC (Gilson Medical Electronics, France) by an automatic sample injector (Gilson model 231). The detection was carried out electrochemically with a carbon electrode, an Ag-AgCl reference electrode and an amperometric detector (Gilson model 141) operated at 0.75 V. The lower limit of noradrenaline detection ranged from 0.35 to 0.50 pmol.

RT-PCR

Total RNA was isolated from mouse colonic epithelium using the Qiagen RNeasy kit (Qiagen). First-strand cDNA was synthesized by reverse transcription of the RNA using Superscript II (Invitrogen) and random hexamer primers (200 ng). PCR was performed using the following sets of primers (5' to 3'): Kir6.1 (GenBank™ accession number D88159) forward nucleotides 126-145, reverse 1041-1022; Kir6.2 (D50581) forward 243-262, reverse 1066-1047; SUR1 (L40624) forward 2091-2110, reverse 2622-2603; SUR2A/B (D86038) forward 4237-4256, reverse 5076-5057 for SUR2A and 4900-4881 for SUR2B. PCR was performed using the hot-start method. 10% of the reverse transcription reaction was combined with 1 μM of each primer, 0.2 mM of each dNTP, 0.75 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 U of Taq polymerase, and autoclaved ddH_2O to a final volume of 20 μl. After 3 min at 94°C, amplifications proceeded for 35 cycles (94°C, 30 s; 58°C, 60 s; 72°C, 3 min) with a final elongation period at 72°C for 7 min. PCR products were separated and visualized on an ethidium bromide-stained 1.5% agarose gel by electrophoresis. The expected sizes (bp) of the PCR products were: 916 (Kir6.1), 824 (Kir6.2), 532 (SUR1), 840 and 664 (SUR2A and SUR2B, respectively).

Chemicals

Amiloride (10 mM), clonidine (1 mM), ouabain (10 mM), rauwolscine (1 mM) and tetrapentylammonium chloride (TPA, 100 mM) were dissolved in H_2O. Furosemide and IBMX were prepared as a 100 mM stock solution in H_2O with a drop of 1N NaOH. Charybdotoxin was prepared as a 10 μM stock in KH solution containing 0.1% BSA. Bumetanide, clotrimazole, forskolin, medetomidine, phentolamine, tolbutamide and UK 14,304 were made as at least 1,000-fold stock solutions in ethanol. Diazoxide (300 mM) and prazosin (10 mM) were dissolved in dimethyl sulphoxide (DMSO). Nystatin was prepared as a 180-mg/ml stock solution in DMSO and sonicated for 30 s just before use. XE991 (a generous gift from Dr. B.S. Brown, DuPont, Wilmington, DE) was prepared as a 10 mM stock solution in 0.1 N HCl. Medetomidine and HMR 1098 were generous gifts from Prof. K. Starke, University of Freiburg, Germany, and Dr. P.E. Light, University of Alberta, Canada, respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Nystatin and diazoxide were prepared fresh before each experiment. Other chemicals were used from stock solutions stored at either 4°C or -20°C.

Data analysis

All data are expressed as means ± SE along with the number of preparations used (N). Statistical difference was determined by Student's *t*-test or one-way ANOVA. Values of $P<0.05$ were considered statistically significant.

RESULTS

Noradrenaline levels in mouse intestine

The total amount of noradrenaline in the proximal colon (including mucosa, submucosa, and muscular layers) was 3.49 ± 0.75 nmol/g (N=5), of which 1.08 ± 0.21 nmol/g (or 31.2 %) was from the epithelial layer. For comparison, mouse jejunum contained 1.70 ± 0.37 nmol/g (N=5), of which 0.04 ± 0.02 nmol/g (or 2.0 %) was from the epithelial layer. The fact that noradrenaline levels in the proximal colonic epithelium are ~20-fold higher than in the jejunal epithelium suggests that α_2ARs could play a significant role in colonic epithelial cell function.

Effect of α_2AR agonists and antagonists on I_{sc}

Application of the specific α_2AR agonist, UK 14,304, produced a concentration-dependent (1 to 10,000 nM, both sides) decrease in the baseline current, with the IC_{50} = 34.7 nM. The effect of UK 14,304 (1 μM) was similar in the presence and absence of 10 μM apical amiloride (-21.5 ± 4.1 and -23.6 ± 3.9 $\mu A/cm^2$, respectively, N=5, P>0.05), indicating that α_2AR activation had no effect on electrogenic Na^+ absorption by ENaC channels. Similar experiments performed in the presence or absence of apical $BaCl_2$ (5 mM) to inhibit K^+ secretion have shown UK 14,304 does not affect K^+ secretion (-18.7 ± 3.6 and -20.1 ± 4.4 $\mu A/cm^2$, N=4, P>0.05). Therefore, all subsequent experiments were performed in the presence of amiloride (10 μM) in the apical compartment. In addition, in Cl^- current studies, $BaCl_2$ (5 mM) was also present in the apical compartment.

α_2AR agonists are more effective inhibitors of I_{sc} in the proximal colon than the distal colon (Fig. 2•1). UK 14,304 (1 μM) decreased the I_{sc} from 40.7 ± 4.4 to 19.6 ± 4.2

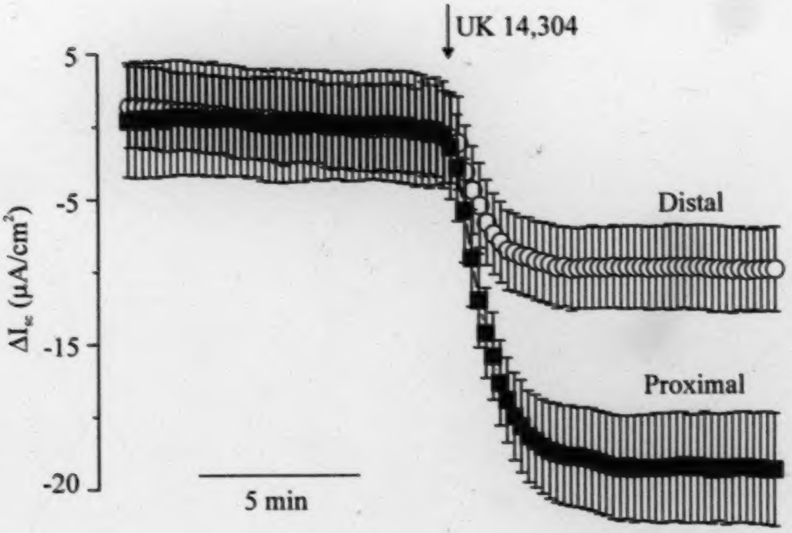

Figure 2·1 Inhibition of I_{sc} by the α_2AR agonist, UK 14,304, in the distal and proximal colon

I_{sc} was more effectively inhibited by UK 14,304 (1 µM, both sides) in the proximal than in the distal colon. The data were obtained in KH solution and show means ± SE from 12 different preparations in each segment. Amiloride (10 µM) and $BaCl_2$ (5 mM) were present in the apical compartment to inhibit Na^+ absorption and K^+ secretion, respectively.

μA/cm^2 (N=14, P<0.001, paired *t*-test) in the proximal colon, and from 34.7 ± 6.3 to 23.9 ± 4.2 μA/cm^2 (N=13, P<0.01, paired *t*-test) in the distal colon. Thus, activation of α_2ARs inhibits ~52% of the proximal and ~31% of the distal colon baseline I_{sc}. Similar results were obtained with two other α_2AR agonists, medetomidine (1 μM, both sides, N=3) and clonidine (10 μM, both sides, N=6; data not shown). The α_2AR antagonists, rauwolscine (1 μM, N=4) and phentolamine (1 μM, N=11), as well as the α_1 adrenoceptor antagonist, prazosin (1 μM, N=4), did not affect the I_{sc} in any segment of the colon. In addition, the presence of prazosin (1 μM, N=4) in the bath solution had no effect on I_{sc} inhibition by UK 14,304. Tissue conductances of the proximal and distal colon in KH solution were 31.1 ± 1.1 mS/cm^2 (N=57) and 20.5 ± 0.9 mS/cm^2 (N=49), respectively, and were not significantly changed by UK 14,304 treatment (data not shown).

α_2ARs inhibit CFTR-mediated Cl$^-$ secretion

Figure 2•2 shows that UK 14,304 (1 μM) inhibited baseline I_{sc} in control but not in CF colonic epithelia. The data are representative of four different experiments with CF tissues. For comparison, the subsequent responses to forskolin and furosemide in both epithelia are also shown. Sequential application of forskolin and furosemide is known to exert opposite effects on I_{sc} in control compared to CF epithelia (7). The results shown in Fig. 2•2 suggest that activation of α_2ARs does not affect forskolin or furosemide responses in either tissue.

Further characterization of the effects of α_2AR agonists on anion secretion has been performed in anion replacement studies. In the proximal colon, in Cl$^-$-free solution, UK 14,304 inhibited I_{sc} by 10.8 μA/cm^2 (from 29.9 ± 3.9 to 19.1 ± 3.6 μA/cm^2, N=4),

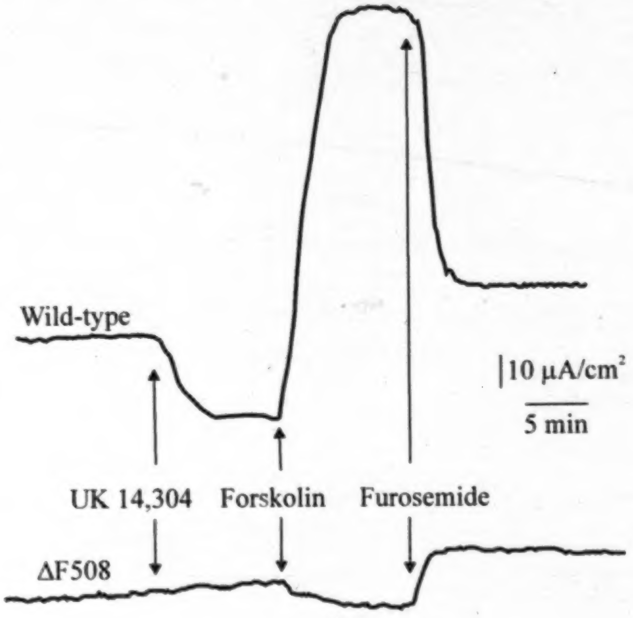

Figure 2·2 Effect of UK 14,304 on I_{sc} in control and CF colonic epithelia
UK 14,304 (1μM, both sides) inhibited baseline I_{sc} in control but not in ΔF508 CF colonic epithelia. For comparison, the responses to forskolin (10 μM, both sides) and furosemide (1 mM, basolateral) in control and CF tissues after UK 14,304 treatment are also shown. The data are representative of four different preparations from CF mice.

whereas in HCO_3^--free solution it was inhibited by 41.6 μA/cm^2 (from 81.3 ± 5.8 to 39.7 ± 4.0 μA/cm^2, N=13). Despite the marked differences in the baseline I_{sc} in these solutions, the relative effect of UK 14,304 in KH and HCO_3^--free solutions was similar (52% vs. 51%), but significantly reduced in Cl^--free solution (~33%). This suggests that UK 14,304 affects mainly Cl^- secretion in colonic epithelia.

A direct way to show that UK 14,304 inhibits Cl^- secretion is to measure chloride movement using $^{36}Cl^-$. Chloride fluxes were measured in both the basolateral to apical and in the apical to basolateral directions. The change in chloride movement in each direction in response to UK 14,304 (1 μM, both sides) was calculated from the specific activity and expressed as a percentage of the I_{sc} response to UK 14,304, obtained by integrating the area under the I_{sc}-time record. The experiments were unpaired, that is each preparation was used to measure flux in only one direction. The results of these studies show that UK 14,304 decreased Cl^- flux in the basolateral to apical direction, and significantly increased Cl^- backflux (Fig. 2•3). In all experiments, amiloride (10 μM) and $BaCl_2$ (5 mM) were present in the apical compartment to inhibit Na^+ absorption and K^+ secretion, respectively, as described earlier (Duszyk et al., 2001). Overall, the net flux decreased by 0.44 μEq cm^{-2}h^{-1}, which corresponded to 96% of that predicted by the I_{sc}.

α_2ARs control basolateral K_{ATP} channels

Chloride flux measurements and the fact that UK 14,304 has no effect on I_{sc} in CF epithelia indicate that α_2ARs regulate CFTR-mediated Cl^- secretion. Primary control of transepithelial anion secretion occurs at the level of apical anion channels as well as basolateral Na^+/K^+-ATPases, $Na^+/K^+/2Cl^-$ cotransporters and K^+ channels. Each of these

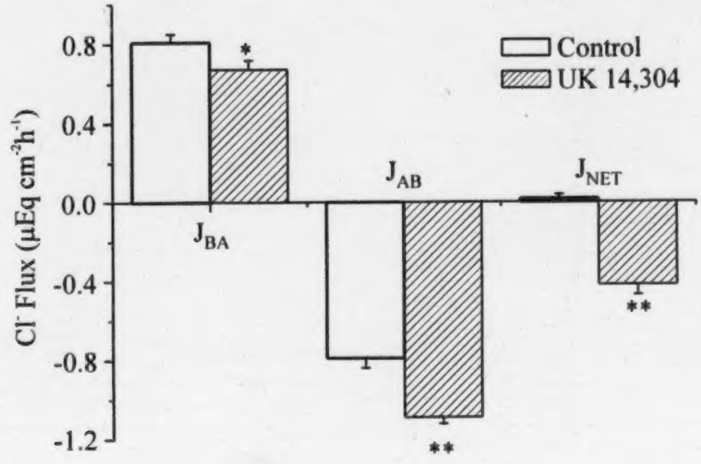

Figure 2·3 Effect of UK 14,304 on Cl⁻ fluxes
Unidirectional and net Cl⁻ fluxes are shown before and after addition of UK 14,304 (1 μM, both sides). Under baseline conditions the basolateral-to-apical (J_{BA}) and the apical-to-basolateral (J_{AB}) fluxes were approximately equal, resulting in a lack of net Cl⁻ flux (J_{NET}). UK 14,304 decreased J_{BA} and increased J_{AB} (N=6 in each direction). In all experiments, amiloride (10 μM) and $BaCl_2$ (5 mM) were present in the apical compartment to inhibit Na^+ absorption and K^+ secretion, respectively. *$P<0.05$, **$P<0.01$

components could be a target for transport modulation. The effects of α_2AR activation on CFTR Cl^- channel function were evaluated by measuring forskolin-activated I_{sc}. Forskolin (10 μM, both sides) increased I_{sc} from 39.2 ± 4.5 to 264.6 ± 2.7 μA/cm^2, N=6. Subsequent application of UK 14,304 reduced I_{sc} by 24.3 ± 8.1 μA/cm^2. Under baseline conditions UK 14,304 reduced the I_{sc} from 36.8 ± 4.1 to 19.9 ± 2.4 μA/cm^2, and subsequent application of forskolin increased I_{sc} by 216.8 ± 7.8 μA/cm^2 (N=6). Statistical analysis of these data indicated that UK 14,304 had similar effects on the baseline and forskolin-activated I_{sc} ($P>0.05$).

An inhibitor of the basolateral $Na^+/K^+/2Cl^-$ cotransporter, furosemide (1 mM), reduced forskolin-activated I_{sc} by ~65%, but had no significant effect on the baseline current (ΔI_{sc} = -3.8 ± 4.1 μA/cm^2, N=6, $P>0.05$ paired Student's *t*-test). In the presence of furosemide, UK 14,304 reduced the I_{sc} by 19.8 ± 3.9 μA/cm^2 (N=4), which is not significantly different from experiments under control conditions. Similar results were obtained with another inhibitor of the basolateral $Na^+/K^+/2Cl^-$ cotransporter, bumetanide (10 μM, N=6, data not shown). These results indicate that α_2ARs do not affect $Na^+/K^+/2Cl^-$ cotransporter function in mouse colonic epithelia.

The effect of α_2ARs on basolateral K^+ channels was assessed in the presence of ouabain (100 μM, basolateral side) to inhibit the Na^+/K^+-ATPase, and with the use of the ionophore nystatin in order to bypass the apical membrane (Fig. 2•4A). In the presence of an apical to basolateral directed K^+ gradient, nystatin (90 μg/ml, apical side) evoked a strong increase in I_{sc}, which rose to a peak value of 325.6 ± 59.8 μA/cm^2 (N=4). When the tissue was pretreated with UK 14,304 (1 μM, both sides) the effect of nystatin was decreased by about half, i.e. the increase in I_{sc} amounted to only 151.6 ± 26.3 μA/cm^2

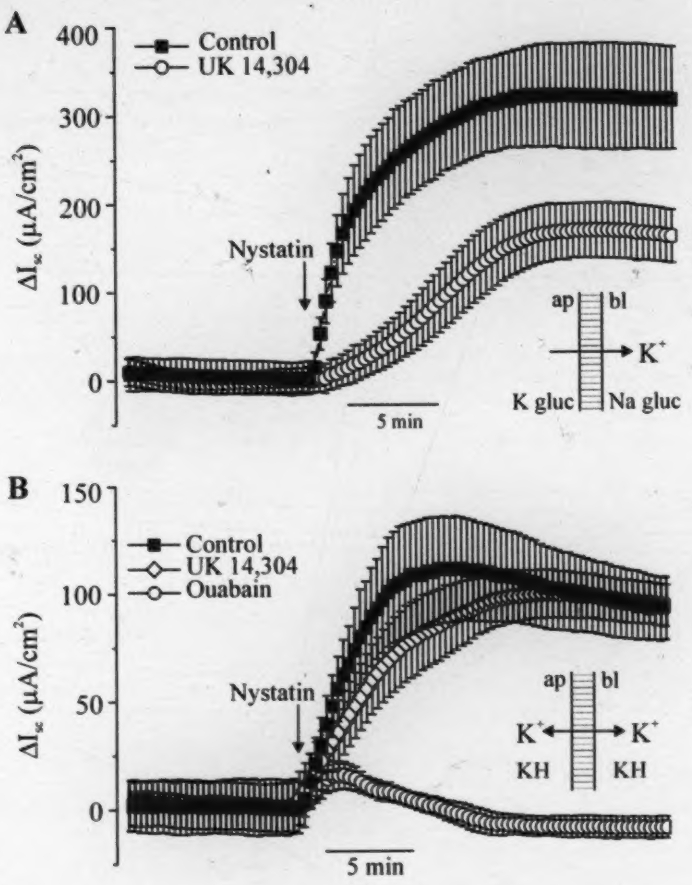

Figure 2·4 Effect of UK 14,304 on nystatin-stimulated I_{sc}

A. Epithelia, pre-treated with ouabain (100 μM, basolateral), were subjected to an apical-to-basolateral K^+ gradient and nystatin (90 μg/ml, apical) was added at the time indicated. B. In the absence of a K^+ gradient, i.e. with standard KH solution on both sides, apical nystatin produced a similar increase in I_{sc} in the absence and presence of UK 14,304 (1 μM, both sides). The effect of nystatin was abolished in the presence of ouabain (100 μM, basolateral), indicating that the nystatin-induced current in the absence of a K^+ gradient is caused by the Na^+/K^+-ATPase. Furthermore, UK 14,304 does not affect Na^+/K^+-ATPase activity. The data are shown as means ± SE from 4 to 7 different experiments. The insets show the direction of K^+ gradients.

(N=4). The effect of UK 14,304 on Na^+/K^+-ATPase activity was investigated in the absence of a K^+ gradient, i.e. with standard KH solution on both sides of the tissue (Fig. 2•4B). Nystatin increased I_{sc} by 108.6 ±16.9 μA/cm^2, and 99.5 ± 10.3 μA/cm^2 in the absence and presence of UK 14,304 (1 μM, both sides), respectively (N=4 in both sets, P>0.05, Student's *t*-test). The effect of nystatin was abolished in the presence of basolateral ouabain (100 μM), indicating that the nystatin-induced current in the absence of a K^+ gradient is caused by the Na^+/K^+-ATPase. Thus, these experiments demonstrate that UK 14,304 inhibits basolateral K^+ channels but not the Na^+/K^+-ATPase. Further experiments were designed to identify the K^+ channels inhibited by α_2ARs.

At least four biophysically and pharmacologically distinct types of K^+ channels contribute to the basolateral K^+ conductance: a cAMP-activated K^+ channel (KCNQ1), an intermediate conductance Ca^{2+}-activated K^+ channel (IK-1), a large conductance Ca^{2+}-activated K^+ channel (BK), and ATP-dependent K^+ channels (K_{ATP}). The KCNQ1 channel can be specifically blocked by the cognitive enhancer XE991 (39), the IK-1 channel - by an antifungal antibiotic, clotrimazole, the BK channel - by charybdotoxin or TPA, and the K_{ATP} channel - by tolbutamide (27). We used the above-mentioned blockers to identify K^+ channels inhibited by α_2ARs. We found that XE991 (30 μM, N=4), clotrimazole (50 μM, N=6), TPA (100 μM, N=5), and charybdotoxin (50 nM, N=4), did not affect I_{sc} inhibition by UK 14,304. However, in the presence of tolbutamide (100 μM), I_{sc} inhibition by UK 14,304 was reduced by 46.2 ± 5.4% (N=8), suggesting the involvement of K_{ATP} channels. In another study we used the specific K_{ATP} opener, diazoxide (300 μM), to study its effects on I_{sc} inhibition by α_2AR agonists. Diazoxide increased I_{sc} by 15.2 ± 2.8 and by 82.5 ± 10.4 μA/cm^2, in KH and HCO_3^--free solutions, respectively (N=4). The diazoxide-activated I_{sc} could be completely inhibited by UK 14,304, confirming the critical role of K_{ATP} channels in inhibition of anion secretion by α_2ARs (Fig. 2•5).

K_{ATP} channels are heteromultimers composed of inwardly rectifying K^+ channel subunits (Kir6.x) and sulfonylurea receptors (SURs) that associate in a 4:4 stoichiometry to form an octameric unit. Glibenclamide has been shown to inhibit K_{ATP} channels containing the SUR1 isoform at nanomolar concentrations, whereas inhibition of channels containing either SUR2A or SUR2B subunits required significantly higher concentrations (28). We found that glibenclamide (200 nM, basolateral) changed the baseline current from 32.7 ± 5.8 to 29.9 ± 4.0 $\mu A/cm^2$ (N=6, $P>0.05$, paired Student's *t*-test), indicating that the K_{ATP} channel in mouse colonic epithelium does not contain the SUR1 isoform. We have not used glibenclamide at higher concentrations, where this chemical is also known to inhibit CFTR. However, it has recently been shown that the sulphonylurea derivative HMR 1098 is a selective inhibitor of K_{ATP} channels containing the SUR2A subunit (26). We used this chemical to investigate the composition of K_{ATP} channels in mouse colonic epithelia. HMR 1098 (20 μM, basolateral) changed the baseline I_{sc} from 35.2 ± 9.9 to 33.8 ± 7.8 $\mu A/cm^2$ (N=6, $P>0.05$, paired Student's *t*-test), indicating that epithelial K_{ATP} channels do not contain the SUR2A isoform. This result is consistent with I_{sc} activation by diazoxide, which is known to activate K_{ATP} channels, containing SUR1 or SUR2B but not SUR2A subunits (1;25).

Further identification of the epithelial K_{ATP} channel composition was performed using RT-PCR studies. Figure 2•6 shows the presence of Kir6.1, Kir6.2, SUR1 and SUR2B in mouse colonic epithelia. SUR2A mRNA was not detected.

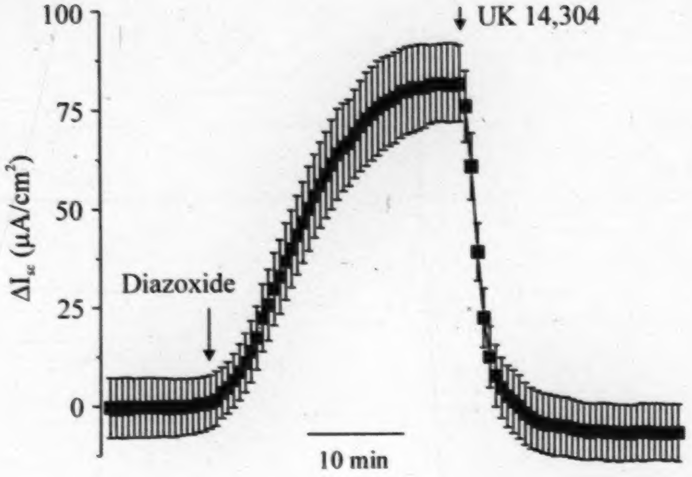

Figure 2·5 Activation and inhibition of K_{ATP} channels in colonic epithelia
Diazoxide (300 μM, basolateral) causes a strong activation in I_{sc}, which is completely inhibited by UK 14,304 (1 μM, both sides). The data show average responses (mean ± SE) from four experiments in HCO_3^--free solution.

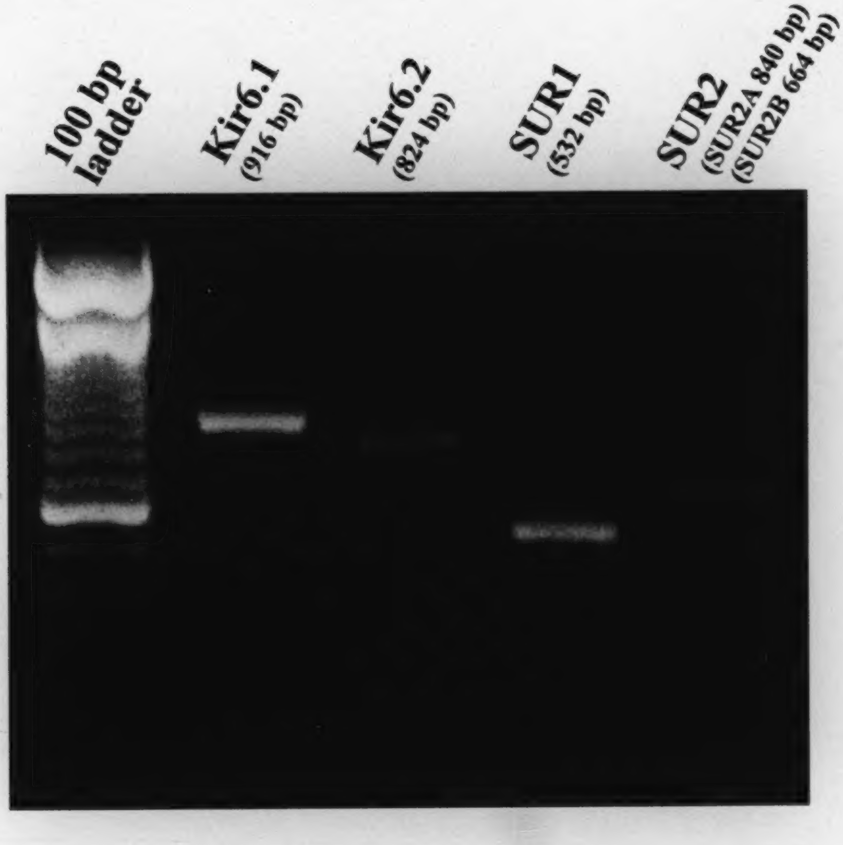
100 bp ladder
Kir6.1 (916 bp)
Kir6.2 (824 bp)
SUR1 (532 bp)
SUR2 (SUR2A 840 bp) (SUR2B 664 bp)

Figure 2·6 Expression of K_{ATP} channel subunits in murine colonic epithelial cells
RT-PCR experiments show the expression of Kir6.1, Kir6.2, SUR1, and SUR2B but not SUR2A mRNAs. The marker lane contains a 100 bp ladder, with the characteristic thick band corresponding to 600 bp. The identity of PCR products was confirmed by sequencing.

α_2ARs inhibit anion secretion by activating $G_{i/o}$ proteins

In many cell types, α_2ARs transduce their signal through pertussis-toxin (PTX) sensitive $G_{i/o}$ proteins. In order to investigate their role in inhibition of Cl^- secretion by α_2ARs, epithelia were incubated with PTX (500 ng/ml, 2 hours) before application of UK 14,304. In two mouse colon preparations PTX completely prevented I_{sc} inhibition by UK 14,304 (1 μM, both sides). However, in three other preparations less than 45% of the UK 14,304 response was reduced by PTX. The reason(s) for this considerable variability between animals, which have been maintained under the same conditions and are otherwise normal, is not certain. Overall, UK 14,304 reduced the I_{sc} by 5.2 ± 1.6 μA/cm^2 (N=14) after PTX treatment. This indicates that more than 75 % of the UK 14,304 response is sensitive to PTX.

Studies with cultured human colonic epithelial cells have shown that both cAMP and Ca^{2+} signaling pathways mediate inhibition of Cl^- secretion by α_2ARs (20;40). Therefore, we used radioimmunoassay to measure cyclic AMP concentrations following exposure of colonic epithelia to either UK 14,304 or to forskolin in the presence or absence of isobutylmethylxanthine (IBMX). The cyclic AMP content of the epithelial tissue plus that released into the medium were measured. We found that UK 14,304 had no significant effect on the intracellular level of cAMP in mouse colonic epithelium (Fig. 2•7A). To measure if α_2ARs affected $[Ca^{2+}]_i$, we used Fura-2 as a reporting molecule and challenged isolated colonic cells with UK 14,304 or ionomycin (Fig. 2•7B). UK 14,304 treatment changed $[Ca^{2+}]_i$ from 242.8 ± 57.1 nM to 251.2 ± 53.2 nM (N=6 mice, $P>0.05$, paired Student's *t*-test). Subsequent application of ionomycin increased $[Ca^{2+}]_i$ to 754.8 ± 171.3 nM.

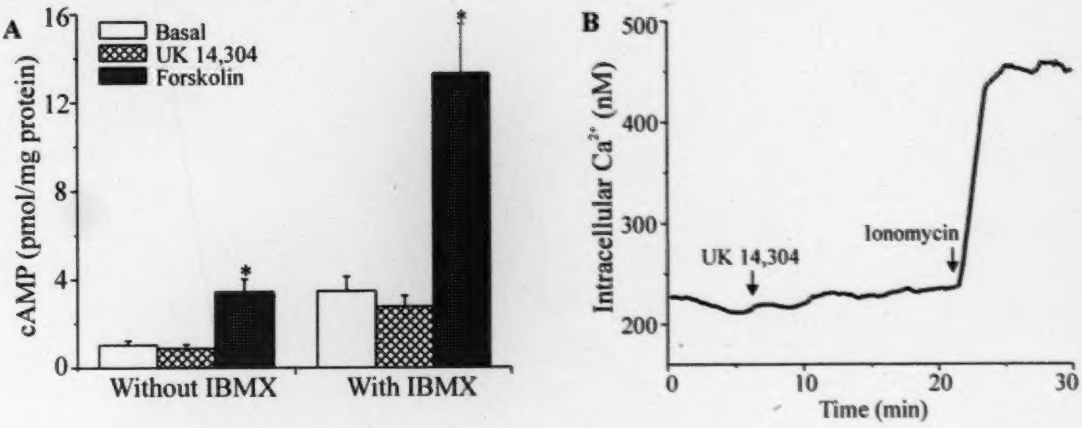

Figure 2·7 cAMP and intracellular Ca^{2+} are not involved in the signal transduction pathway activated by UK 14,304
A: The amount of cAMP refers to that present in the tissue and that lost into the medium under basal conditions, during UK 14,304 (1 μM) exposure, or during forskolin (10 μM) exposure, in the presence or absence of IBMX (100 μM). Forskolin was used as a positive control. Values are means of 4 triplicate measurements ± SE. B: UK 14,304 (1 μM) has no effect on [Ca^{2+}]$_i$. Ionomycin (10 μM) was used as a positive control. The data are representative of six different preparations. *P<0.05 single-tail Student's *t*-test, compared with the basal values.

DISCUSSION

Earlier studies have shown that human intestinal mucosa possessed the highest α_2AR density in the proximal colon, and that their number decreased gradually towards the distal section (36). Our study shows that α_2AR agonists inhibited Cl^- secretion with the greatest potency in the proximal section, suggesting a similar gradient of α_2AR distribution in the mouse colon.

The α_2AR family is comprised of three subtypes of receptors, α_{2A}, α_{2B} and α_{2C}, encoded by distinct genes (17). The rodent α_{2A}AR differs pharmacologically from the human α_{2A}AR, and is sometimes called α_{2D}AR (5). Studies of α_2AR subtype tissue distribution have shown that intestinal epithelia express α_{2A}AR but not α_{2B}AR or α_{2C}AR (29;36). These receptors are present in the basolateral membrane of colonic epithelial cells (36). The α_2ARs have differential sensitivity to prazosin: α_{2A}AR are insensitive ($K_i > 300$ nM), α_{2B}AR are very sensitive ($K_i = 5$ nM), and α_{2C}AR have an intermediate sensitivity ($K_i = 15$-36 nM) (4). The fact that in our studies prazosin did not affect I_{sc} inhibition by UK 14,304 supports the conclusion that α_{2A}ARs mediate the effects of UK 14,304 in colonic epithelial cells.

Experiments with CF mice, Cl^- flux measurements, and ion replacement studies indicated that in mouse colonic epithelium α_2ARs inhibit transepithelial Cl^- secretion. Other studies have shown that α_2AR agonists inhibit cAMP-dependent Cl^- secretion in human colon (20) and rat jejunum (38), but activate K^+ secretion in rat colon via Ca^{2+}-dependent pathways (21;30). This indicates that the effect of α_2AR activation varies amongst species and tissues and may involve activation of different targets and signal transduction pathways.

Intestinal Cl^- secretion depends on the coordinated activity of apical Cl^- channels as well as basolateral $Na^+/K^+/2Cl^-$ cotransporters, Na^+/K^+-ATPases and K^+ channels. Inhibition of one of these transport mechanisms leads to the inhibition of Cl^- secretion. Our data show that in mouse colon α_2ARs affect Cl^- secretion by inhibiting basolateral K_{ATP} channels, in a process that likely requires activation of $G_{i/o}$ proteins, but does not involve intracellular second messengers such as cAMP and Ca^{2+}.

A number of K^+ channels have been identified on the basolateral membrane of mammalian colon (2). Interestingly, our studies show that, of these, only K_{ATP} channels are affected by α_2ARs. These channels are constitutively active, since their blockers inhibit baseline I_{sc} by more than 50%. K_{ATP} channels are heteromultimers composed of inwardly rectifying K^+ channel subunits (Kir6.x) and sulfonylurea receptors (SURs) that associate in a 4:4 stoichiometry to form an octameric K_{ATP} channel (1). Various combinations of these two subunits convey the heterogeneity in channel properties observed in native cells such as Kir6.2/SUR1 in pancreatic β-cells and neural tissue, Kir6.2/SUR2A in cardiac and skeletal muscles, and Kir6.1/SUR2B or Kir6.2/SUR2B in vascular smooth muscle (1). RT-PCR experiments and pharmacological studies have shown that colonic epithelial cells do not express the SUR2A subunit, and the lack of sensitivity to glibenclamide suggests that the epithelial K_{ATP} channel complex does not contain SUR1. Thus, K_{ATP} channels in colonic epithelia are likely to be similar to vascular smooth muscle K_{ATP} channels, composed of SUR2B and either Kir6.1 or Kir6.2 subunits.

α_2ARs are members of the G protein-coupled receptor superfamily that interact primarily with $G_{i/o}$ proteins (6). Most (but not all) physiological signaling pathways linked to α_2AR activation involve these PTX-sensitive G-proteins. For example, α_{2A}ARs were shown to inhibit adenylyl cyclase upon stimulation by UK 14,304 in a PTX-resistant manner (41), and to have the potential to couple physically and functionally to other G proteins (11). In addition, during the past few years several reports have described various physiological consequences of GPCR stimulation that were not mediated by G protein activation (for review see (18)). In our experiments, pretreatment of cells with PTX reduced I_{sc} inhibition by α_2AR agonists by ~75%. This suggests that interaction with $G_{i/o}$ proteins is an integral part of α_2AR function in colonic epithelium, but does not exclude a possibility that some effects of α_2AR activation could be mediated via a $G_{i/o}$ protein-independent pathway.

The regulation of K_{ATP} channels by $G_{i/o}$ proteins has been demonstrated by several authors (13;22;24). These studies showed that PTX-sensitive $G_{i/o}$ proteins activated K_{ATP} channels in pancreatic cells (13), cardiac myocytes (24), and neurons (14). Interestingly, our data show that in murine colonic epithelium activation of $G_{i/o}$ proteins by α_2ARs inhibits K_{ATP} channels. This result is consistent with studies showing that α_2ARs inhibit Cl^- secretion via activation of PTX-sensitive G proteins (40), and that blockers of K_{ATP} channels reduced anion secretion in human colonic epithelial cells (27).

ATP inhibits K_{ATP} channels with IC_{50} values generally in the low micromolar range, 5 – 20 μM (1). Since the intracellular ATP concentration in most cells lies between 3 and 7 mM, it may be argued that K_{ATP} channels should never be open under ATP-rich, physiological conditions. Interestingly, our data show that K_{ATP} channels are tonically active in the colonic epithelium. This fact suggests that other regulatory mechanisms are involved in the control of K_{ATP} channel activity. One such mechanism may involve increased levels of MgADP, which has been shown to activate K^+ channels inhibited by ATP (9;23). Another mechanism may involve phosphatidylinositol phosphates, which facilitate channel activity in the presence of ATP by antagonizing the ATP-induced channel inhibition (3;32). In addition, there is significant evidence that supports the existence of subcellular compartmentalization in different cells (33). This suggests that not all ATP could gain access to K_{ATP} channels, limiting effective ATP concentration in the vicinity of the channel.

In conclusion, our results provide new insights into the mechanisms of α_2AR regulation of anion secretion. We have shown that α_2ARs inhibit anion secretion in colonic epithelia by acting on basolateral K_{ATP} channels, through a process that does not involve classical second messengers, such as cAMP or Ca^{2+}. This type of regulation may be of clinical and pharmacological relevance in understanding the molecular mechanisms of diseases characterized by abnormal intestinal secretion.

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CHAPTER 3

Membrane cholesterol content modulates activation of BK channels in colonic epithelia

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INTRODUCTION

The plasma membrane of eukaryotic cells contains a far greater variety of lipid species than is required to form a lipid bilayer, suggesting that lipids play a unique and determining role in membrane organization (6). In recent years studies on lipid biophysics, protein sorting and detergent solubility have converged to support the concept that lipids are not homogeneously distributed within the lipid bilayer but spontaneously aggregate to form microdomains enriched in cholesterol and sphingolipid (32;37). The physical characteristics of these microdomains or "lipid rafts" are proposed to preferentially admit and retain proteins modified by unsaturated long chain fatty acids while excluding the majority of transmembrane proteins (37;38). Thus by accumulating signaling molecules and selectively admitting transmembrane proteins, lipid rafts have the potential to compartmentalize signals within the plasma membrane (39). A potential problem with the lipid raft concept is that most of the evidence for the existence of lipid rafts derives from indirect approaches such as resistance to detergent extraction at sub-physiological temperature, and the use of cholesterol-sequestering agents. This has led some investigators to question the existence of lipid rafts and to offer alternative explanations for the effect of modulating cholesterol and sphingolipids on cell function. For example, it was recently suggested that the major role of cholesterol and sphingolipids might be to regulate the permeability of the exofacial leaflet of the bilayer (28). Therefore, while there is consensus that lipids modify cellular functionality in reproducible ways, the mechanisms responsible have yet to be fully resolved.

In the last few years several reports have provided evidence that certain ion channels physically associate with the low-density detergent-insoluble microdomains that characterize lipid rafts. For example, both cardiac (42) and epithelial (16) Na^+ channels were recovered within such fractions. Similarly, the voltage-gated K^+ channels, Kv1.5 and Kv2.1 (but not channel Kv4.2) (25;26), and the large conductance Ca^{2+}-activated K^+ (BK) channels (4) were observed within cholesterol-enriched microdomains. Associations with lipid rafts have been shown to involve the transport of Kir3.1/3.2 channels to the membrane (9), and to control the activities of both Cl^- (3) and Ca^{2+} (5) channels. Thus, although the functional implications of channel associations with plasma membrane microdomains are not well understood, there is growing evidence that these structures play an important role in the modulation of ion channel activity. The localization of ion channels within lipid rafts is an attractive concept, which would integrate ion channels into multi-component signaling complexes capable of generating localized signals in the plasma membrane.

The role of lipid rafts in intestinal epithelial cell function has been explored in several studies. Cholesterol-enriched microdomains were shown to play a role in cholesterol trafficking (10), and in apical exocytotic membrane trafficking in enterocytes (12;13). However, very little is known about the role of these microdomains in transepithelial anion transport. Chloride secretion in colonic epithelia drives water into the intestinal lumen, providing for the fluidity of intestinal contents (2). Either over-secretion or under-secretion of chloride can result in significant pathophysiological events, such as secretory diarrhea or cystic fibrosis, respectively. Therefore, significant efforts have been made to study anion secretion in order to understand the underlying mechanisms involved.

The aim of our study was to characterize the role of cholesterol-enriched microdomains in the regulation of transepithelial anion secretion. Using a combination of biophysical, pharmacological and biochemical approaches we have investigated the role of cholesterol and plasma membrane microdomains in transepithelial anion secretion in colonic epithelia. Our studies show that lowering the cholesterol and sphingomyelin content of the basolateral plasma membrane leads to the activation of BK channels, to an increase in the detergent solubility of BK channels, and to the stimulation of transepithelial anion secretion. Our results therefore identify a novel cholesterol-dependent mechanism of BK channel regulation operative in colonic epithelia. BK channels were not detected within conventional low-density detergent-insoluble microdomains, but were associated with a high-density detergent-insoluble pellet containing the cytoskeleton. Since BK channels and the raft associated protein caveolin both translocated into a high-density detergent-soluble phase on reducing cholesterol we propose that BK channels may be the first ion channel shown to associate with a cytoskeletally anchored lipid raft.

MATERIALS AND METHODS

Epithelial cells

The colonic epithelia were from three different strains of mice: BALB/c, C57BL/6J and cystic fibrosis (CF) mice. The breeding colony of CF mice (B6.129S6-Cftr^{tm1Kth}, Jackson Laboratory, Bar Harbor, ME) was housed in a pathogen-free environment (Health Sciences Laboratory Animal Services, University of Alberta). All experiments were carried out with the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta. Pups were weaned at 21 days of age, and genotyped as described previously (19). Mice were killed by CO_2 narcosis, and 6 cm-long pieces of colon were removed from ~2 cm below the caecum and immediately placed in cold Krebs-Henseleit solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. The colons were opened up, the muscle layers dissected away and four pieces were mounted in Ussing chambers with a 0.2 cm^2 recording area.

Transepithelial measurements

Ussing chamber experiments were performed as described earlier (19). For basolateral membrane K^+ current measurements, apical NaCl was replaced by equimolar potassium gluconate, while basolateral NaCl was substituted with sodium gluconate and the Ca^{2+} concentration was increased to 5 mM in both solutions, to compensate for the Ca^{2+}-buffering capacity of gluconate. In addition, 100 μM ouabain was added to the basolateral compartment to inhibit the Na^+/K^+-ATPase. Subsequent permeabilization of the apical membrane with nystatin (90 μg/ml), allowed measurement of K^+ current as these ions moved down their concentration gradient through basolateral K^+ channels.

Lipid raft protein isolation and immunoblotting

Isolated epithelium was treated with or without 10 mM MβCD for 30 min (in KHS, bubbled with $95\%O_2/5\%CO_2$) and then lysed in 1 ml of cold MES-buffered saline (25 mM MES, 150 mM NaCl, 5 mM $Na_4P_2O_7{\cdot}10H_2O$, 1 mM $NaVO_4$, 2 mM NaF, pH 6.5, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, pepstatin) for 90 min at 4°C. The lysate (containing 200 μg of protein) was loaded with an equal volume of 90% sucrose and overlaid with 30% and 5% sucrose.

Centrifugation was performed at 165,000 RCF (relative centrifugal force) for 18 hours at 4°C in a Beckman SW60Ti rotor. Starting from the top of the gradient 9 fractions, including the pellet, were collected and separated by SDS-PAGE (5-20% gradient gel). The proteins were transferred to nitrocellulose membranes incubated with 5% (w/v) non-fat milk powder in phosphate-buffered saline plus Tween 20 (PBST: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.05% (v/v) Tween 20, pH 7.2), washed and incubated with polyclonal rabbit antibodies against the BK channel α subunit (Chemicon International, Temecula, CA) diluted 1:500, or with polyclonal rabbit antibodies against caveolin-1 (Santa Cruz Biotech., Santa Cruz, CA) diluted 1:200. The membranes were incubated with 1:10,000 peroxidase-conjugated goat anti-rabbit IgG (H+L, Jackson ImmunoResearch Laboratory, West Grove, PA) and then washed in PBST. Detection was carried out using the ECL kit (Amersham Biosciences, Buckinghamshire, England) and Hyperfilm™ (Amersham Biosciences).

Chemicals

Amiloride (10 mM), $BaCl_2$ (500 mM), ouabain (10 mM) and tetrapentylammonium chloride (TPeA, 100 mM) were dissolved in H_2O, methyl-β-cyclodextrin (MβCD) and mannitol (80 mM) in KHS, diphenylamine-2-carboxylate (DPC, 1 M) and pimaric acid (10 mM, a generous gift from J. Clay, Helix Biotech, New Westminster, BC) in dimethyl sulphoxide (DMSO). Furosemide (100 mM) was prepared in H_2O with a drop of 1N NaOH, charybdotoxin (10 μM) in KHS containing 0.1% BSA. Bumetanide, ceramide, clotrimazole, and tolbutamide were made as at least 1,000-fold stock solutions in ethanol. Filipin (10 mg/ml in methanol) and nystatin (90 mg/ml in DMSO) were prepared fresh before each experiment. XE991 (10 mM, a generous gift from Dr. B.S. Brown, DuPont, Wilmington, DE) was dissolved in 0.1 N HCl. $BaCl_2$ and mannitol were from Fisher Scientific (Fair Lawn, NJ), all other chemicals were from Sigma-Aldrich.

Statistical analysis

Data are expressed as means ± SE with the number of preparations used (N). Statistical difference was determined by Student's *t*-test or one-way ANOVA. Values of $P<0.05$ were considered statistically significant.

RESULTS

Basolateral lipid rafts affect anion secretion in colonic epithelia

The cyclic oligosaccharide methyl-β-cyclodextrin (MβCD) is a membrane impermeable molecule that selectively and rapidly extracts cholesterol from the plasma membrane. We applied MβCD to either the apical or basolateral side of epithelial cell sheets from colonic epithelia and measured the effect on transepithelial short-circuit current (I_{sc}). Figure 3•1A shows that in KHS, apical MβCD (10 mM, N=11) had no significant effect on I_{sc} for at least 30 min ($P>0.05$, Student's *t*-test). In contrast, basolateral MβCD (10 mM, N=38) produced a biphasic I_{sc} response, with an initial peak followed by an elevated plateau. When MβCD was added unilaterally, mannitol (10 mM) was added to the opposite side to compensate for changes in osmotic pressure. Mannitol by itself had no effect on I_{sc} (N=9, $P>0.05$). Bilateral MβCD produced a change in I_{sc} that was identical to that caused by basolateral MβCD (N=8). MβCD had no effect on transepithelial resistance for at least 90 min ($P>0.05$, paired Student's *t*-test, N=12),

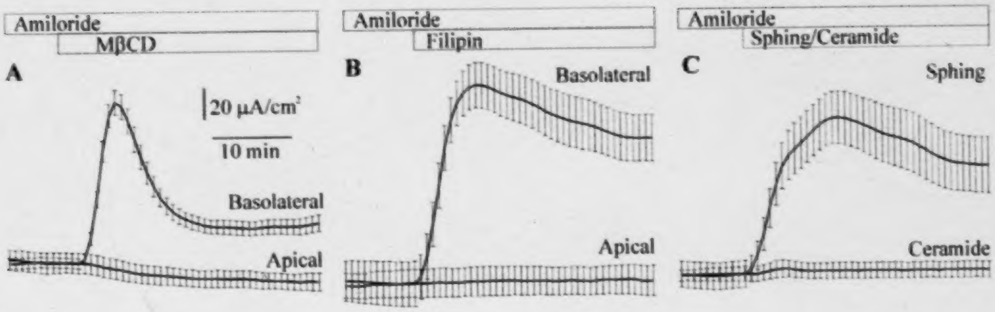

Figure 3·1 The effects of MβCD, filipin and sphingomyelinase on I_{sc}

A: Basolateral MβCD (10 mM, N=38) produced a biphasic I_{sc} response, with an initial increase followed by a plateau that was attained after ~ 20 min. Apical MβCD (N=11) had no significant effect on I_{sc} for at least 30 min. Both filipin (B, 10μg/ml, N=8) and neutral sphingomyelinase (C, 1.6 U/ml, N=6) activated I_{sc} from the basolateral side only. The product of sphingomyelinase metabolism, ceramide (10 μg/ml, both sides) had no effect on I_{sc} (N=6, P>0.05, Student's *t*-test). In all experiments, 10 μM amiloride was present in the apical compartment to inhibit Na^+ channels.

indicating that this treatment does not affect the paracellular pathway. Similarly, it did not affect cell viability, since the relative I_{sc} activated by the cAMP- or Ca^{2+}-dependent secretagogues (10 μM forskolin and 100 μM carbachol, respectively) was not altered by MβCD treatment (10 mM, basolateral, 90 min; Table 3•1). Moreover, tetrodotoxin (5 μM, data not shown) did not affect the response to basolateral MβCD, ruling out the contribution of residual neural activity. The effect of MβCD was also not affected by the presence of the epithelial Na^+ channel (ENaC) blocker, amiloride (10 μM, N=12, P>0.05). Therefore, all subsequent experiments were performed with amiloride in the apical compartment to inhibit ENaC-mediated Na^+ absorption.

Filipin is a cholesterol-binding reagent that is frequently used in lipid raft studies. We found that apical filipin (10 μg/ml, N=4) had no effect on the baseline I_{sc} (Fig. 3•1B). However, basolateral filipin (N=8) increased I_{sc} similarly to MβCD, although the peak was broader and the plateau level was attained at higher I_{sc} (Fig. 3•1B).

Lipid raft integrity depends on the presence of both cholesterol and sphingomyelin. We reasoned that treating cells with sphingomyelinase would alter raft structure or composition and affect ion flux mediated by these domains, if they were involved in this process. Apical sphingomyelinase (1.6 U/ml) had no effect on the baseline I_{sc} (N=4), but basolateral sphingomyelinase produced a change in I_{sc} that was qualitatively similar to the effects of MβCD and filipin (Fig. 3•1C). One of the products of sphingomyelinase action is ceramide. Therefore, we applied ceramide (10 μg/ml, both sides) to investigate if the effect of sphingomyelinase could be related to ceramide production. Figure 3•1C shows that ceramide had no effect on I_{sc} (N=6, P>0.05, Student's *t*-test). The fact that three dissimilar treatments that affect lipid raft integrity produced qualitatively similar

Table 3•1

I_{sc} activated by forskolin and carbachol under control and MβCD-pretreated conditions

	Control ΔI_{sc} ($\mu A/cm^2$)	N	MβCD (10 mM) ΔI_{sc} ($\mu A/cm^2$)	N
Forskolin (10 μM, bilateral)	322.4 ± 9.6	13	326.8 ± 16.0	7
Carbachol (100 μM, basolateral)	183.0 ± 10.6	15	182.4 ± 16.7	10

N - number of experiments

effects on I_{sc}, suggests that these microdomains are involved in the regulation of ion transport in epithelial cells.

Lipid raft disruption affects CFTR-mediated anion secretion

In order to identify the current affected by cholesterol depletion, we used colonic epithelium from ΔF508CFTR mice. Figure 3•2A shows that MβCD has no significant effect on I_{sc} in CF mice (N=6). Similarly, the CFTR Cl^- channel blocker, DPC (0.5 mM, apical, N=8), significantly inhibited I_{sc} activation by MβCD. These experiments indicate that disruption of lipid rafts affects CFTR-mediated anion secretion.

Anion secretion in epithelial cells is coordinated by a network of ion channels, transporters, and energy-dependent pumps that are selectively expressed in the apical or basolateral aspects of the epithelium. Since MβCD, filipin and sphingomyelinase affected I_{sc} from the basolateral side only, we focused on this side to identify the target(s) that were affected by these reagents.

Basolateral BK channels are associated with lipid rafts

An inhibitor of the basolateral $Na^+/K^+/2Cl^-$ cotransporter, furosemide (1 mM), had no effect on the baseline current (ΔI_{sc} = -1.6 ± 2.1 μA/cm^2, N=4, P>0.05 Student's *t*-test), and in its presence MβCD increased the I_{sc} by 92.8 ± 13.6 μA/cm^2 (N=4), which is not significantly different from control conditions. Similar results were obtained with another inhibitor of the basolateral $Na^+/K^+/2Cl^-$ cotransporter, bumetanide (10 μM, N=6). These results indicate that cholesterol depletion does not affect $Na^+/K^+/2Cl^-$ cotransporter

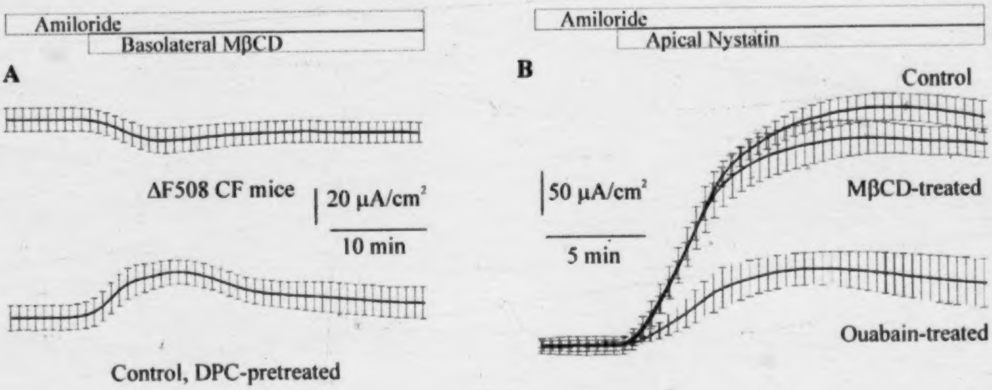

Figure 3·2 MβCD treatment affects CFTR-mediated anion secretion
(A) MβCD did not activate I_{sc} in ΔF508 CF colonic epithelia (N=6), and DPC (0.5 mM, apical) blocked the effect of MβCD (10 mM, N=8) on I_{sc} in wild-type mice. (B) MβCD treatment has no effect on I_{sc} generated by the Na^+/K^+-ATPase in nystatin-permeabilized epithelia. The data are means ± SE from 3 to 4 recordings.

function in colonic epithelia.

The effect of MβCD on Na^+/K^+-ATPase activity was investigated with the use of the ionophore nystatin in order to bypass the apical membrane (Fig. 3•2B). In regular Krebs-Henseleit solution, nystatin (90 μg/ml, apical) activated similar currents in the presence or absence of MβCD (10 mM, N=4 in both sets, P>0.05, Student's *t*-test), indicating that removal of cholesterol from the basolateral side does not affect Na^+/K^+-ATPase function in colonic epithelia. The effect of nystatin was inhibited by basolateral ouabain (100 μM, N=3), confirming that the nystatin-induced current is generated by the Na^+/K^+-ATPase.

The effect of MβCD on basolateral K^+ channels was assessed in nystatin-permeabilized epithelia in the presence of ouabain (100 μM) to inhibit the Na^+/K^+-ATPase, and an apical-to-basolateral directed K^+ gradient (Fig. 3•3A). Nystatin (90 μg/ml, apical) increased I_{sc} from 72.6 ± 19.8 $\mu A/cm^2$ (N=4) under control conditions to 125.6 ± 21.3 $\mu A/cm^2$ (N=4, P<0.05 Student's *t*-test) in MβCD-treated epithelia (Fig. 3•3A), indicating that removal of cholesterol from the basolateral membrane activated K^+ channels in colonic epithelia. Further studies were designed to identify the K^+ channels activated by MβCD.

At least four biophysically and pharmacologically distinct types of K^+ channels contribute to the basolateral K^+ conductance in mammalian colon: a cAMP-activated K^+ channel (KCNQ1) (24), an intermediate conductance Ca^{2+}-activated K^+ channel (IK-1) (7), a large conductance Ca^{2+}-activated K^+ channel (BK) (24), and ATP-dependent K^+ channels (K_{ATP}) (19). The KCNQ1 channel can be specifically blocked by the cognitive enhancer XE991, the IK-1 channel - by an antifungal antibiotic, clotrimazole, the BK channel - by Ba^{2+}, charybdotoxin or TPeA, and the K_{ATP} channel - by tolbutamide (27).

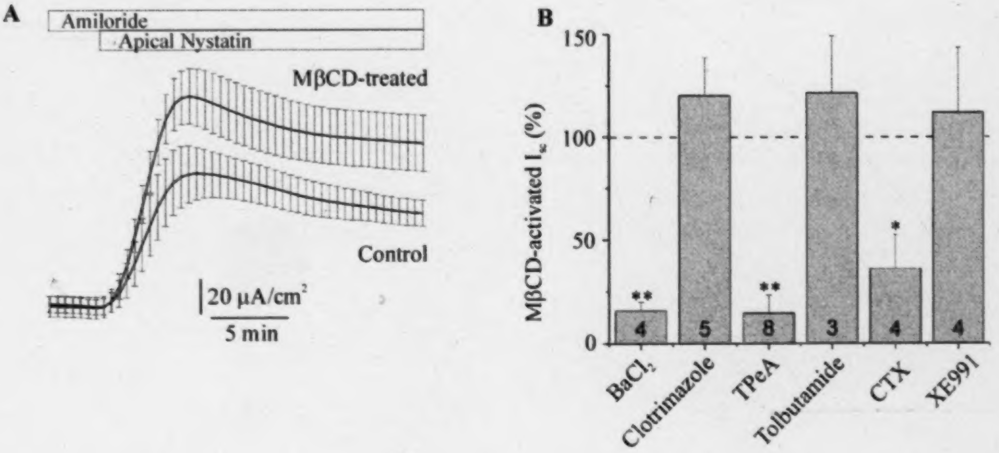

Figure 3·3 Activation of basolateral K^+ channels by MβCD treatment
Epithelia pre-incubated with ouabain (100 μM, basolateral), were subjected to an apical-to-basolateral K^+ gradient and nystatin (90 μg/ml, apical) was added at the time indicated (A). The data are shown as means ± SE from 4 control and 7 MβCD-treated I_{sc} measurements. B: The effect of K^+ channel blockers on I_{sc} activation by MβCD. Epithelia were pre-treated from the basolateral side with $BaCl_2$ (5 mM), clotrimazole (50 μM), TPeA (100 μM), tolbutamide (100 μM), charybdotoxin (CTX, 100 nM) or XE991 (30 μM), before MβCD treatment (10 mM, basolateral). Values are expressed as percentages of MβCD-activated I_{sc} in the absence of K^+ channel blockers. The number of experiments is shown in the histogram. *$P<0.05$, **$P<0.01$.

We used the above-mentioned blockers to identify the K^+ channels activated by MβCD. We found that XE991 (30 μM, N=4), clotrimazole (50 μM, N=5), and tolbutamide (100 μM, N=3), did not affect I_{sc} activation by MβCD (Fig. 3•3B). However, in the presence of TPeA (100 μM, N=4) I_{sc} activation by MβCD was reduced by 85.5 ± 8.7% (N=4, P<0.01, paired Student's *t*-test). Similar results were obtained using charybdotoxin and $BaCl_2$ (Fig. 3•3B), suggesting that BK channels were activated by cholesterol removal in colonic epithelia. This conclusion has been further supported by experiments with the specific BK channel opener, pimaric acid (17). Figure 3•4 shows that I_{sc} activation by pimaric acid (90 μM, N=8) was significantly attenuated after pretreatment of the epithelium with MβCD. The I_{sc} peak response to pimaric acid was reduced from 65 $\mu A/cm^2$ under control conditions to 12.5 $\mu A/cm^2$ after MβCD treatment.

In subsequent studies we used rabbit polyclonal antibodies against the BK channel α subunit to investigate BK channel expression in colonic epithelia. Western blot experiments have shown that this antibody recognized a ~125 kDa protein band that disappeared after pre-absorption of the primary antibody with a blocking peptide supplied by the manufacturer (data not shown). This antibody was used to detect BK channels in fractions obtained after sucrose density gradient centrifugation. Figure 3•5 shows that the BK channel is present mainly in the detergent-insoluble pellet that contains the actin cytoskeleton. The gradient fractions were also blotted for the caveolae marker, caveolin-1, which was found not only in the low buoyant density raft fractions 4-5, but also in higher density fractions including the pellet. Interestingly, after MβCD treatment the majority of BK channels translocated from the pellet to lighter density fraction 8 which contains detergent-soluble proteins (Fig. 3•5). The translocation of BK channels from the

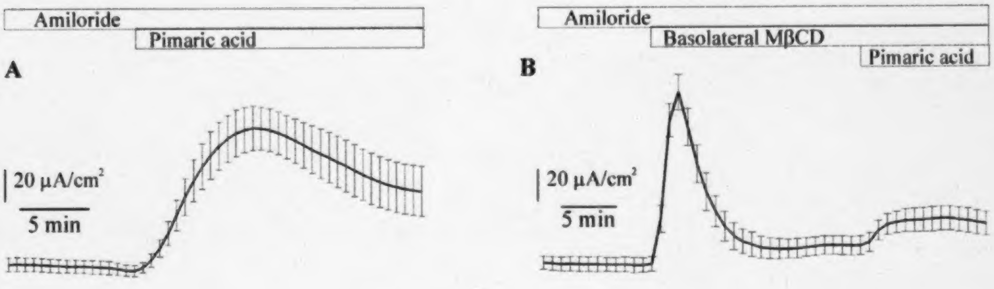

Figure 3·4 The response to pimaric acid in control and MβCD-treated epithelia
Activation of I_{sc} by pimaric acid (90 µM, both sides) in control (A, N=6) and MβCD-treated (B, 10 mM, N=8) epithelia.

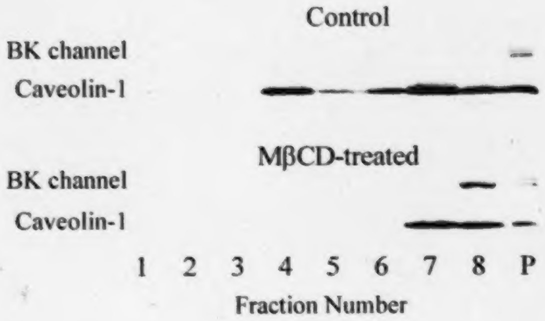
Control
BK channel
Caveolin-1
MβCD-treated
BK channel
Caveolin-1
1 2 3 4 5 6 7 8 P
Fraction Number

Figure 3·5 BK channels partition in the pellet fraction of sucrose density gradients and do not co-localize with caveolin in colonic epithelial cells
Cells were lysed in MES-buffered saline + 1% Triton X-100 and fractionated in sucrose gradients. Fractions were collected from the gradient top (1) to bottom (8), where P is the pellet, and analyzed by immunoblotting with the indicated antibodies. For MβCD treatment, cells were incubated for 30 minutes with 10 mM MβCD before lysis. The BK channel antibody recognizes the C-terminal region of the α subunit, and the expected size is 125 kDa. The caveolin-1 antibody recognizes a 24 kDa protein.

pellet was accompanied by a similar shift in the buoyant density of caveolin, which also accumulated in fractions 7 and 8. Since MβCD treatment displaced caveolin from the low buoyant density fractions, it was effective in disrupting caveolin-containing lipid rafts. Thus our evidence indicates BK channels are selectively associated with the cytoskeleton and not within the low-density lipid rafts, but are nevertheless dependent upon cholesterol for anchorage to the cytoskeleton. This suggests that BK channels are associated with a subset of lipid rafts that are selectively anchored to the cytoskeleton.

DISCUSSION

The main finding of our study is that BK channels, present in the basolateral membrane of colonic epithelial cells, control transepithelial anion secretion through a cholesterol-dependent mechanism. The convergent effects of cholesterol depletion by MβCD, filipin and of sphingolipid depletion by sphingomyelinase provide strong evidence that lipid microdomains in the plasma membrane play an important role in the regulation of anion secretion.

Lipid rafts in intestinal epithelial cells have been extensively characterized (8;10;12). The apical (brush border) membrane contains at least two different types of lipid raft microdomains that are different from rafts present in the basolateral membrane or on the surface of other cell types (8). In particular, apical rafts are stable rather than transient, and their core components include glycolipids as well as the divalent lectin galectin-4 (8). Although apical rafts contain cholesterol, it is not essential for raft stability (12). The results of our studies show that the removal of cholesterol from the apical membrane, or treatment with the sphingomyelin-degrading enzyme, sphingomyelinase, has no effect on transepithelial anion secretion. Since the majority of apical anion conductance in colonic epithelia is mediated by CFTR (24), this suggests that raft integrity is not essential for CFTR function.

Anion secretion in epithelial cells depends on the coordinated activity of apical Cl^- channels, as well as basolateral Na^+/K^+-ATPases, $Na^+/K^+/2Cl^-$ cotransporters and K^+ channels. The role of rafts in the regulation of Na^+/K^+-ATPase is controversial. Several studies have shown that caveolae do not contain Na^+/K^+-ATPase (13;21), whereas others have suggested that Na^+/K^+-ATPase is present in lipid rafts (22). These contradictory conclusions were based on the biochemical analysis of proteins found in caveolae, and could be related to differences in the experimental methods used to isolate cell membrane microdomains (e.g. in the presence or absence of Triton X-100). Our results indicate that disruption of basolateral rafts has no effect on Na^+/K^+-ATPase activity in colonic epithelia. In addition, pharmacological studies showed that the $Na^+/K^+/2Cl^-$ cotransporter does not play a role in the response to basolateral lipid raft disruption.

A number of distinct K^+ channels have been identified on the basolateral membrane of mammalian colon (2). In our earlier studies we have shown that K_{ATP}, but not BK channels, were tonically active and controlled baseline anion secretion in murine colonic epithelia (19). The results of this study further expand those observations by showing that lipid rafts control the activity of basolateral BK channels. Under baseline conditions these channels are found mainly in the high-density pellet fraction, and are inactive, since charybdotoxin has no effect on the baseline I_{sc}. Following MβCD treatment they translocate out of the pellet fraction and become active, resulting in cell hyperpolarization and stimulation of anion secretion. The fact that pimaric acid-induced BK channel activation is inhibited after MβCD treatment suggests that BK channels may either be fully activated by cholesterol removal, or that pimaric acid activates only those BK channels that are associated with lipid rafts.

Although lipid rafts are by definition recovered within low buoyant density fractions, subsets of lipid rafts may associate with the cytoskeleton. For example multivalent ligation of the Fc receptor in neutrophils results in translocation into high buoyant density lipid rafts (1;34). Other studies have shown that a subset of plasma membrane skeleton proteins co-isolate with cholesterol-rich, detergent-resistant membrane fragments that exhibit a high buoyant density in sucrose (29). Similarly clustering the hyaluronan receptor CD44 promotes association with lipid rafts and the redirection of actin bundles into the raft (30). Mass spectrometric analysis of lipid raft proteomes, including our own studies, identify structural components of the intermediate filament and actin cytoskeleton as frequent components of low-density detergent-insoluble fractions (11;20;29;36;40;41). Actin is a common component of such fractions and clustering raft-based glycolipids or GPI-linked proteins promotes local organization of F-actin (14). Similarly cortical F-actin spikes originate within the caveolae of adipocytes (18). Thus although their exact roles remain to be determined we have speculated that lipid rafts regulate cytoskeletal assembly and conversely that cytoskeletal assemblies with lipid rafts might provide submembranous scaffolds for the assembly and function of signaling molecules (36). According to this view the functions of lipid rafts and the assembly of cortical cytoskeletal assemblies are reciprocally linked. If lipid rafts are closely associated with F-actin assembly then those rafts, which are most tightly associated with the cytoskeleton, might be expected to segregate with the cytoskeletal pellet following detergent extraction. In the current study failure to identify BK channels within the low buoyant density fractions demonstrates that BK channels are not associated with conventional lipid rafts. However, translocation of BK channels from the cytoskeleton to the detergent-soluble fraction on lowering cholesterol would be compatible with the disruption of cytoskeletally-associated lipid rafts, and with the redistribution of caveolin from both low- and high-density fractions to those of intermediate density. The absence of BK channels from low-density domains indicates that if BK channels associate with lipid rafts they do so only as a very late event and may therefore be components of a subset of lipid rafts that associate exclusively with the cytoskeleton.

Many studies of lipid rafts have been conducted in transformed cell lines that are non-polarized. In polarized epithelia the cytoskeleton is most elaborated on the basolateral margin. It therefore seems likely that apically located lipid rafts would be recovered within the low buoyant density fractions, whereas basolaterally located rafts would associate with the cytoskeleton. The cytoskeleton can provide a scaffold for the assembly, juxtaposition and three-dimensional orientation of signaling complexes. The mechanism of BK channel activation by MβCD is presently unknown. However, the fact that under control conditions BK channels are found in the pellet fraction suggests that they are constitutively present in large macromolecular complexes that maintain the channel in the closed state. Cholesterol removal could cause the breakup of these structures leading to BK channel translocation into a lighter fraction and simultaneous removal of inhibitory interactions that hold the channel in the closed state. A similar mechanism has been recently proposed for adenylyl cyclase activation, based on the observation that treatment with MβCD augmented the enzyme activity (35). Lipid rafts are also known to contain proteins that are involved in Ca^{2+} homeostasis (5;22), and changes in $[Ca^{2+}]_i$ due to lipid raft disruption could lead to BK channel activation. However, a recent study has shown that basal levels of $[Ca^{2+}]_i$ in MβCD-treated and untreated cells were identical (31).

The physiological significance of BK channel regulation by lipid rafts, in particular cholesterol, is presently unknown. However, *in vivo* studies show that feeding mice with *n*-3 polyunsaturated fatty acids may reduce cell membrane cholesterol content in colonic epithelia by 46%, without altering total cellular levels (23). The results of this study indicate that such a change would have a major effect on transepithelial anion secretion in the colon. Similarly, changes in membrane cholesterol concentration could be related to reverse cholesterol transport, the process whereby cholesterol is removed from peripheral tissues and is delivered to the liver for subsequent excretion into bile (15). The ability of peripheral cells to participate in reverse cholesterol transport has been proposed to be essential in establishing the proper cholesterol distribution in cells. Measurements of the cholesterol efflux from epithelial cells have shown that cholesterol efflux occurs less readily from the apical than basolateral membrane (33), a result consistent with the observed effect of cholesterol removal on I_{sc} reported in this study. In summary, we have shown that lipid raft disruption stimulates transepithelial anion secretion by a mechanism compatible with the activation of basolateral BK channels in colonic epithelial cells. These channels are of particular interest because they transform intracellular signals into changes in membrane conductance, and are thought to be loci where the regulation of anion secretion is accomplished.

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CHAPTER 4

Cholesterol-dependent regulation of basolateral adenosine-mediated anion secretion in colon epithelial cells

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INTRODUCTION

Adenosine, a purine nucleoside, is a known regulator of epithelial ion channel function. Both potassium (15;36) and chloride conductances (8;35) appear to be under the regulation of adenosine in a variety of epithelia, and in mammalian intestinal epithelium it is well known that adenosine activates anion secretion (2;10;15;34). There are 4 subtypes of the G protein-coupled adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3. A_1 and A_3 receptors are coupled to $G_{i/o}$ and $G_{i/q}$ proteins, respectively, whereas A_{2A} and A_{2B} receptors are coupled to G_s proteins and activate adenylyl cyclase (AC) and cAMP production. It is well established that adenosine stimulates Cl^- secretion in colonic epithelial cells (34). Sources of adenosine may be at the apical or basolateral membrane of intestinal epithelial cells, and so receptors appear to be present in both membranes (2;34). Intestinal inflammation is characterized by an influx of inflammatory cells, and adenosine receptors likely play an immunological role by altering secretion in response to inflammation-induced adenosine production from neutrophils (24), eosinophils (29), and mast cells (1). High concentrations of adenosine may also be present in the case of bacterial-induced secretory diarrhea. Killing a large amount of enterogenic bacteria will result in a significant amount of ATP release in the gut lumen, and ATP can be metabolized to adenosine, whose actions have implications in secretory diarrhea.

Adenosine metabolism and homeostasis is under the control of metabolic enzymes and nucleoside transporters. The surface enzyme ecto-5'-nucleotidase (also called CD73), which is present in intestinal epithelial cells (33), produces adenosine from 5'-AMP. Adenosine also has autocrine or paracrine actions, and after its production it can then act on its receptors or be transported into the cell. Cellular scavenging of adenosine occurs through the nucleoside transporters, of which there are two families: the concentrative, or Na^+-dependent, nucleoside transporters (CNTs) and the equilibrative, or Na^+-independent, nucleoside transporters (ENTs). The two families of nucleoside transporters have several subtypes, and currently there are four ENT and three CNT isoforms characterized. Studies looking at nucleoside transporters in colon epithelial cells have shown the presence of ENTs (25;37). Ward and Tse showed that both ENT1 and ENT2 were functionally expressed, however neither CNT1 nor CNT2 was present (37). In a study looking at mRNA expression, it appears that CNT1 is absent, CNT2 and CNT3 are present in low levels, and ENT1, 2, and 3 are present in the mouse colon (23). Once inside the cell, adenosine metabolism is due to the actions of adenosine kinase and adenosine deaminase.

The cell membrane contains the majority of total cell cholesterol, where one of its functions is to act as a regulator of membrane proteins. It has long been known that membrane transport is affected by cell membrane cholesterol (32), and in intestinal epithelium, the function of a variety of membrane proteins appears to rely on cholesterol (5;12;20). Cholesterol has been identified as a backbone in the structure of membrane microdomains known as lipid rafts. Rafts contain certain membrane proteins and cholesterol may exert control of proteins through raft-mediated mechanisms. Receptors, including adenosine receptors (21;31), G proteins and other signaling molecules, and their effectors, including ion channels (30), have been found to associate with rafts. Although the effects of adenosine on ion transport have been widely studied, little is known about the mechanisms regulating adenosine-mediated epithelial ion transport in the intestine.

For these studies, we explored the relationship between cell membrane cholesterol and adenosine-mediated ion transport. We found that altering basolateral, but not apical, cell membrane cholesterol levels affected adenosine receptor activation. This effect was not due to lipid raft disruption, since the cholesterol-binding drug filipin and the sphingomyelin-depleting drug sphingomyelinase did not affect adenosine receptor activation. More specifically, caveolae are also not involved since the responses of adenosine receptor activation on tissue from caveolin-1-deficient mice and wildtype mice were indistinguishable. Anisotropy experiments showed that filipin and sphingomyelinase do not affect cell membrane fluidity, however, the cholesterol-depleting drug methyl-β-cyclodextrin increased cell membrane fluidity. In addition, the effect was not due to a contribution from nucleoside transporters. We show here that modulating basolateral membrane cholesterol levels affected the I_{sc} response to basolateral A_{2A} receptor activation, and this corresponded to a basolateral intermediate conductance Ca^{2+}-regulated K^+ (IK) channel current.

MATERIALS AND METHODS

Epithelial cells

The colonic epithelia used in this study were from three different strains of mice: C57BL/6J, cystic fibrosis (CF), and caveolin-1 knockout mice. The breeding colony of CF mice (B6.129S6-*Cftr*^{tm1Kth}, Jackson Laboratory, Bar Harbor, ME) was housed in a specific pathogen-free environment (Health Sciences Laboratory Animal Services, University of Alberta). Pups were weaned at 21 days of age, and genotyped as described previously (19). Caveolin-1-deficient mice (STOCK Cav^{tm1Mls}/J) were purchased from

Jackson Laboratory and a breeding colony was maintained under the same conditions as the CF mouse colony. All experiments described in this study were carried out with the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta. Mice were killed by CO_2 narcosis, and 6 cm-long pieces of colon were removed from ~2 cm below the caecum and immediately placed in cold Krebs-Henseleit solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. The colons were opened up, and the muscle layers were dissected away. Pieces of 0.2 cm^2 were taken from the colon and mounted in Ussing chambers.

Transepithelial measurements

Ussing chamber short-circuit current (I_{sc}) experiments were performed as described earlier (19). To compensate for changes in osmotic pressure, 10 mM mannitol was added to the apical side at the same time of basolateral MβCD addition. Tissue integrity was monitored throughout the experiments by transepithelial resistance measurements from 0.5 mV pulses every 90 seconds.

RT-PCR

Total RNA was isolated from mouse colonic epithelium using the Qiagen RNeasy kit (Qiagen). First-strand cDNA was synthesized by reverse transcription of the RNA using Superscript II (Invitrogen) and random hexamer primers (200 ng). PCR was performed using the following sets of primers (5' to 3'): mENT1 forward nucleotides 537-564, reverse 1018-992; mENT2 forward 735-754, reverse 1137-1116. PCR was performed using the hot-start method. 10% of the reverse transcription reaction was combined with 1 μM of each primer, 0.2 mM of each dNTP, 0.75 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 U of Taq polymerase, and autoclaved ddH_2O to a final volume of 20 μl. After 3 min at 94°C, amplifications proceeded for 30 cycles (94°C, 30 s; 54°C, 60 s; 72°C, 3 min) with a final elongation period at 72°C for 7 min. PCR products were separated and visualized on an ethidium bromide-stained 1.5% agarose gel by electrophoresis. The expected sizes (bp) of the PCR products were: 482 (mENT1), 403 (mENT2).

Steady-state fluorescence anisotropy

Anisotropy measurements experiments were performed on a PTI fluorimeter equipped with manual polarizers (PTI, London, Ontario, Canada), by estimating fluorescence polarization of 1-(4-trimethyl-ammoniumphenyl)6-phenyl-1,3,5-hexatriene (TMA-DPH). TMA-DPH inserts into the cell membrane and its relative motion is determined by polarized fluorescence expressed as the anisotropy constant, which is inversely proportional to the degree of membrane fluidity (18). Excitation and emission wavelengths were set at 350 nm and 434 nm with a slit-width of 1 nm and 4 nm, respectively. Colonic epithelial cells were incubated at 37°C with 1 μM TMA-DPH (final concentration) in the dark in KHS for 20 min. Unincorporated dye was removed by repeated washings with KHS. Labeled cells were suspended at a concentration of $2x10^6$/ml in KHS, and fluorescence intensity was measured with the excitation polarizer in the vertical position and the analyzing emission polarizer in both the vertical (I_{VV}) and horizontal (I_{VH}) positions. Anisotropy, r, was calculated using the equation:

$r = \dfrac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$. The *G* factor (=$I_{HV}/I_{HH}$) was calculated using horizontally polarized excitation and subsequent comparison of the horizontal and vertical emissions, which for our machine is 0.881. High degrees of fluorescence anisotropy indicate higher degrees of membrane order or lower degrees of membrane fluidity. Cholesterol measurements were performed using the Amplex® Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions.

Chemicals

Adenosine (100 mM), amiloride (10 mM), and N^6-cyclopentyladenosine (CPA) were dissolved in ddH_2O. Methyl-β-cyclodextrin (MβCD, 80 mM), mannitol (80 mM) and water-soluble cholesterol in KHS. Clotrimazole and forskolin, were made as at least 1,000-fold stock solutions in ethanol. Dipyridamole, 2-*p*-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680) and 3-propyl-6-ethyl-5-[(ethylthio)carbony]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS-1523) were dissolved in DMSO. S-(4-Nitrobenzyl)-6-thioinosine (NBTI) and filipin (10 mg/ml, prepared fresh before each experiment) were dissolved in methanol. XE991 (10 mM, a generous gift from Dr. B.S. Brown, DuPont, Wilmington, DE) and 5'-N-ethylcarboxamidoadenosine (NECA, 10 mM) were dissolved in 0.1 N HCl. The stock solution of 1-(4-trimethyl-ammoniumphenyl)6-phenyl-1,3,5-hexatriene (2 mM TMA-DPH in DMSO) was stored at -20°C. Mannitol was from Fisher Scientific (Fair Lawn, NJ), all other chemicals were from Sigma-Aldrich.

Data analysis

All data are expressed as means ± SE along with the number (N) of preparations used. Statistical difference was determined by Student's *t*-test or one-way ANOVA. Values of $P<0.05$ were considered statistically significant.

RESULTS

Basolateral adenosine receptor activation stimulates anion secretion

Basolateral adenosine receptor agonists activate I_{sc} in colonic epithelium. Figure 4•1A shows that 10 μM basolateral NECA (an adenosine analog that is not metabolized or transported) activates I_{sc}. Basolateral NECA activated I_{sc} by 6.3 ± 1.0 μA/cm^2, N=12 (Fig 4•2) and basolateral adenosine (100 μM) activated I_{sc} by 13.5 ± 2.8 μA/cm^2, N=10 (Fig 4•2). CGS-21680 is a high affinity selective agonist for the A_{2A} receptor (13). Basolateral addition of 1 μM CGS-21680 also activated I_{sc} by 8.1 ± 7.2 μA/cm^2, N=4 (Fig 4•2). Apical adenosine receptors are present since apical adenosine activated I_{sc} (100 μM, 8.0 ± 4.6 μA/cm^2, N=4). Similarly, apical NECA also activated I_{sc} (10 μM, 4.1 ± 2.2 μA/cm^2, N=5).

Equilibrative nucleoside transporters are present in colonic epithelium (23;37), however few studies have addressed their activity in this tissue. To look at the basal activity of the ENTs, the general ENT inhibitor dipyridamole (100 μM bilateral, 10 min) was used, however, it did not have a large effect on basal I_{sc} (2.8 ± 0.7 μA/cm^2, N=5). Similarly, addition of the ENT1-selective inhibitor nitrobenzylthioinosine (NBTI, 20 μM bilateral, 10 min) also did not have a large effect on basal I_{sc} (2.3 ± 0.1 μA/cm^2, N=4).

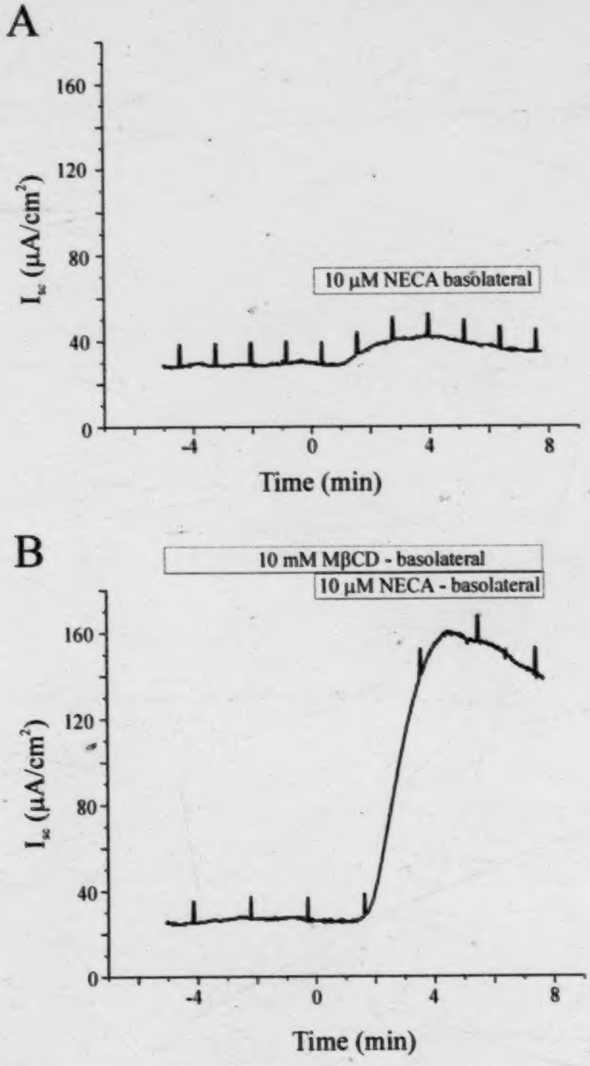

Figure 4·1 The effect of basolateral NECA on colonic I_{sc} in control (A) or MβCD-treated (B) conditions

The data shown are representative I_{sc} traces. Transepithelial resistance was monitored by 0.5 mV pulses applied approximately every 90 seconds.

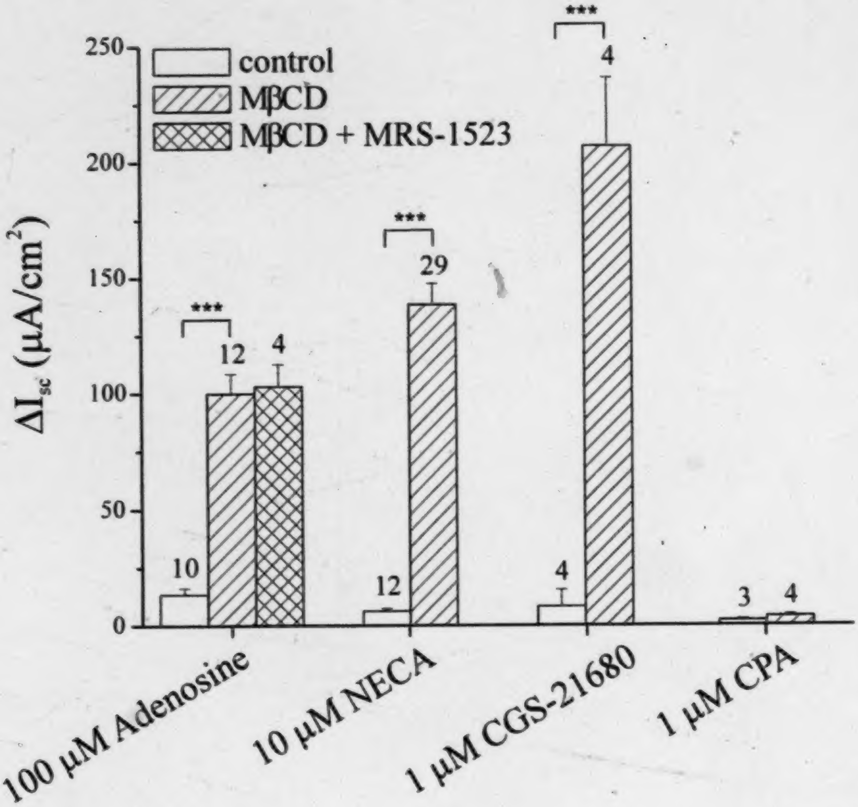

Figure 4·2 The effect of basolateral adenosine receptor agonists on control and cholesterol-depleted colonic epithelia

MβCD (10 mM) refers to pretreatment of the basolateral membrane for 90 minutes before addition of the adenosine receptor agonist. MβCD + MRS-1523 refers to pretreatment with MβCD, as before, and then treatment with the A_3 receptor antagonist MRS-1523 (1 μM basolateral, 10 min). CGS-21680 is a selective agonist for A_{2A} receptors and CPA is a selective agonist for A_1 receptors. The data shown are means ± SE. *** P<0.001

Basolateral cell membrane cholesterol removal enhances adenosine A_2 receptor-activated anion secretion by a lipid raft-independent mechanism

Basolateral addition of MβCD (10 mM, 90 min), which selectively removes membrane cholesterol, caused an increased response to the subsequent addition of certain basolateral adenosine receptor agonists. Figure 4•1B shows a I_{sc} trace of the response to basolateral NECA after MβCD treatment. The responses to basolateral adenosine (100 μM, 99.9 ± 8.6 μA/cm^2, N=12), basolateral NECA (10 μM, 138.3 ± 9.1 μA/cm^2, N=29), and basolateral CGS-21680 (1 μM, 206.9 ± 23.4 μA/cm^2, N=4) were all significantly greater after basolateral cholesterol removal compared to their respective control responses ($P<0.001$, Student's *t*-test, Fig 4•2). If MβCD was added to the apical side, the response to apical adenosine was not significantly greater than in the control conditions (100 μM, 8.2 ± 3.8 μA/cm^2, N=3, $P>0.05$).

Pretreatment with the selective A_3 receptor antagonist MRS-1523 (1 μM basolateral, 10 min) did not block the MβCD-enhanced adenosine response (103 ± 9.5 μA/cm^2, N=3, $P>0.05$, Fig 4•2). To assess if the A_1 receptor was involved, the selective A_1 receptor agonist CPA was used. Figure 4•2 shows that the response to CPA (1 μM basolateral) was not significantly different between control (2.0 ± 0.6 μA/cm^2, N=3) and MβCD-treated conditions (3.8 ± 0.8 μA/cm^2, N=4, $P>0.05$). These results suggest that the increased response to adenosine receptor stimulation after MβCD treatment is not mediated by the A_1 or the A_3 receptors.

The small, but discernable, effect of the ENT inhibitors NBTI and dipyridamole suggests that ENTs are basally active in colonic epithelium. RT-PCR showed the presence of ENT1, but not ENT2, mRNA (Fig 4•3). A decrease in ENT activity upon

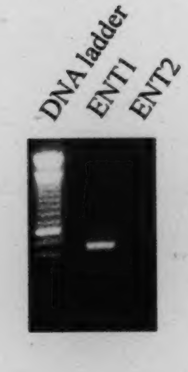

Figure 4·3 Equilibrative nucleoside transporter expression in colonic epithelium
RT-PCR of mENT1 (482bp) and ENT2 (403bp). The DNA ladder is a 100bp ladder with the thicker band corresponding to 600 bp.

cholesterol depletion might contribute to the enhanced adenosine response we observed. The effect of cholesterol removal on the ENT1 was assessed by pretreatment with MβCD before NBTI addition (2.5 ± 0.4 μA/cm^2, N=5, P>0.05). The response to NBTI was not significantly different compared to control conditions, which excludes a significant contribution from altered ENT activity on the MβCD-enhanced adenosine receptor agonist response. Although CNT activity was not assessed, it is unlikely to contribute to the response we were studying since CNTs transport adenosine against its gradient into the cell.

Lipid rafts are membrane microdomains that are rich in cholesterol and sphingolipids, and certain proteins show higher affinities for these areas. MβCD is commonly used to alter lipid raft integrity, as are other drugs that affect cholesterol and sphingolipids. Using the cholesterol-binding drug filipin (10 μg/ml basolateral, 90 min), instead of basolateral MβCD, did not affect the response to basolateral NECA (4.5 ± 2.3 μA/cm^2, N=5, P>0.05, Fig 4•4). In addition, depletion of cell membrane sphingomyelin with sphingomyelinase (1.6 U/ml basolateral, 90 min) did not affect the response to basolateral NECA (8.6 ± 3.2 μA/cm^2, N=4, P>0.05, Fig 4•4). Caveolae are a subset of membrane microdomains, also rich in cholesterol and sphingolipids, and defined by their resident protein caveolin-1. The effect of 10 μM NECA in caveolin-1-deficient mice, which lack caveolae (11), was not significantly different compared to wildtype mice (8.4 ± 0.6 μA/cm^2, N=4, P>0.05, Fig 4•4). Taken together, these results rule out a contribution from membrane microdomains in the enhanced adenosine receptor-activated anion secretion due to cholesterol removal.

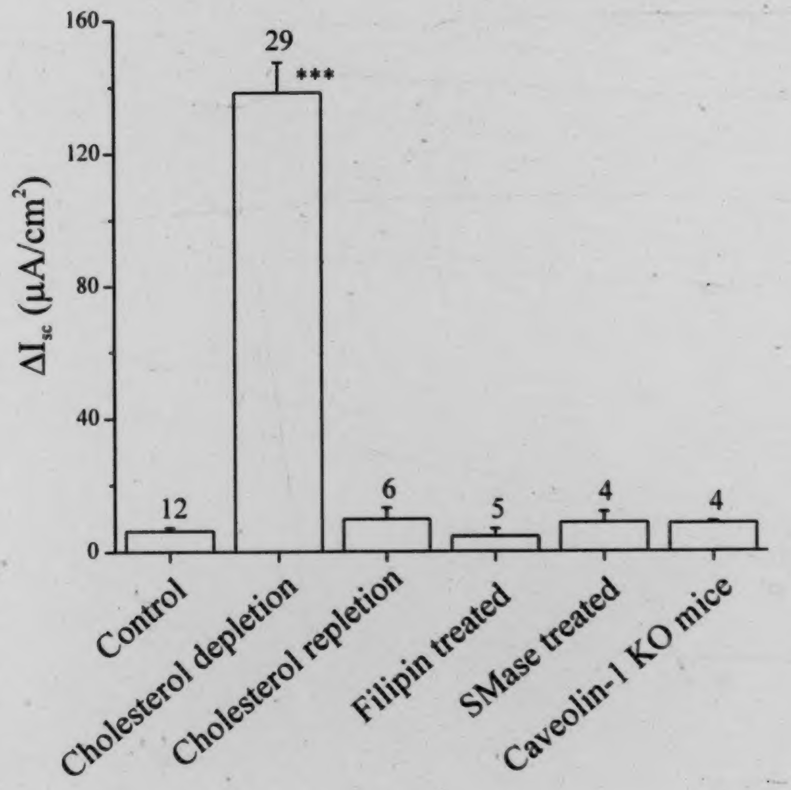

Figure 4·4 Membrane cholesterol content modulates the response to basolateral NECA in colonic epithelia
All experiments looked at the response to 10 μM basolateral NECA. Cholesterol depletion refers to MβCD (10 mM basolateral, 90 min) pretreatment and cholesterol repletion refers to MβCD (10 mM basolateral, 90 min) treatment and subsequent treatment with cholesterol-loaded MβCD (600 μg cholesterol/ml basolateral, 100 min). The basolateral membrane was also pretreated either with filipin (10 μg/ml, 90 min) or sphingomyelinase (SMase, 1.6 U/ml, 90 min). The data shown are means ± SE using one-way ANOVA. *** $P<0.001$

Repletion of cholesterol, after incubation with MβCD (10 mM basolateral, 90 min), was achieved by the addition of water-soluble cholesterol (600 μg cholesterol/ml basolateral, 8 mM MβCD, 100 min) and resulted in a restoration of the NECA response to control levels (9.7 ± 2.4 $\mu A/cm^2$, N=3, P>0.05, Fig 4•4), compared to the response without cholesterol repletion. These studies showed that basolateral cholesterol repletion reverses the effect of cholesterol removal on adenosine receptor activation.

MβCD, but not filipin and sphingomyelinase, affects membrane fluidity

We have used steady-state fluorescence anisotropy measurements to evaluate the effects of MβCD, filipin and sphingomyelinase on cell membrane fluidity. Measurements were performed using the dye 1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) as a marker. The steady-state fluorescence polarization of TMA-DPH represents membrane motional order and is inversely related to membrane fluidity. Figure 4•5 shows a summary of changes in anisotropy following MβCD, filipin and sphingomyelinase treatments. MβCD treatment led to a decrease in the fluorescence anisotropy of TMA-DPH, which suggested changes in the order of the phospholipid molecules. Direct measurements of cholesterol content in mouse colonic epithelium have shown that MβCD (10 mM, 30 min) reduced the cholesterol level from 36 ± 12 μg/mg protein (N=3) to 15 ± 7 μg/mg protein (P<0.05, N=3). Thus, MβCD treatment reduces cholesterol content in epithelial cells leading to an increase in cell membrane fluidity. In contrast, both filipin and sphingomyelinase had no significant effect on cell membrane fluidity (Fig 4•5), indicating that their effects on I_{sc} are independent of cell membrane fluidity.

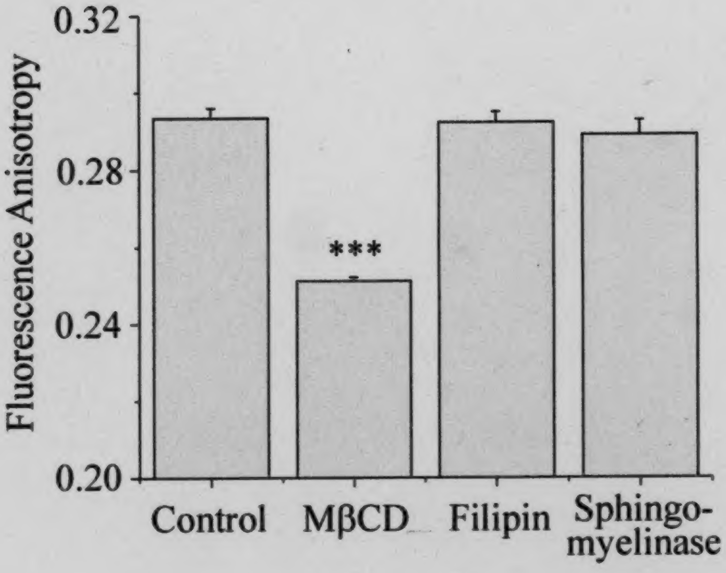

Figure 4·5 Membrane cholesterol depletion increases membrane fluidity in colon epithelial cells
Changes in fluorescence anisotropy values of TMA-DPH measured in cells treated with MβCD (10 mM), filipin (10 μg/ml) or sphingomyelinase (1.6 U/ml). The data shown are means ± SE of three experiments with triplicate determinations. *** $P<0.001$

Anion secretion is due to IK channel activation, not adenylyl cyclase activation, and accounts for the MβCD-induced increase in NECA response

To determine if the enhanced NECA response after cholesterol depletion was activating anion secretion we performed experiments with CF mice, which lack functional CFTR. These studies confirmed that anion secretion was activated since the basolateral NECA response (10 μM basolateral) after cholesterol depletion (10 mM MβCD basolateral, 90 min) was virtually absent (0.4 ± 0.6 μA/cm^2, N=4, $P<0.001$, Fig 4•6) in CF mice, compared to wildtype mice.

Basolateral K$^+$ channels are present in the basolateral membrane of colonic epithelial cells, where they can respond to intracellular Ca^{2+} or cAMP. An activation of basolateral K$^+$ conductance can act as a driving force for Cl$^-$ secretion, and we wanted to see if certain basolateral K$^+$ channels were activated in the enhanced NECA response after cholesterol depletion. We used selective inhibitors of intermediate conductance calcium-activated K$^+$ (IK) channels and cAMP-dependent K$^+$ channels, and an activator of large conductance calcium-activated K$^+$ (BK) channels. IK channels are inhibited by clotrimazole. Pretreatment of the cells with basolateral clotrimazole (90 μM, 10 min), before the addition of basolateral MβCD and then basolateral NECA, significantly reduced the NECA response (19.5 ± 3.1 μA/cm^2, N=8, $P<0.001$, ANOVA, Fig 4•6), compared to treatment without clotrimazole. Similar experiments using an inhibitor of cAMP-dependent K$^+$ channels (30 μM XE991 basolateral) did not significantly affect the response to basolateral NECA after cholesterol depletion (113.0 ± 13.9 μA/cm^2, N=3, $P>0.05$, Fig 4•6). Activating BK channels with pimaric acid (100 μM basolateral) did not affect the response to basolateral NECA after cholesterol depletion (104.3 ± 15.7

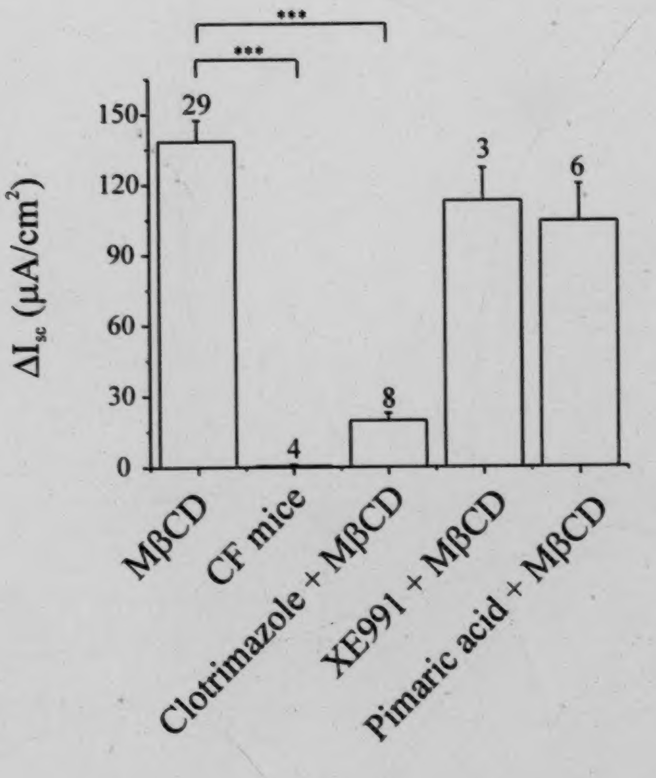

Figure 4•6 The enhanced response to basolateral NECA, after cholesterol depletion, affects anion secretion

The basolateral NECA (10 μM basolateral) response, after MβCD (10 mM) treatment, activates basolateral IK channels and anion secretion. The selective K^+ channel inhibitors, clotrimazole (90 μM basolateral) and XE991 (30 μM basolateral), or the BK channel opener pimaric acid (100 μM basolateral) were added before NECA addition. The data shown are means ± SE. *** $P<0.001$

$\mu A/cm^2$, N=6, $P>0.05$, Fig 4•6). This further suggests that BK channels are not contributing to the cholesterol-sensitive NECA-activated I_{sc}.

The A_{2A} and A_{2B} receptors are coupled to G_s proteins, which activate AC and cAMP production. The response to the AC activator forskolin (10 μM bilateral, 322.0 ± 17.3 $\mu A/cm^2$, N=17) is unchanged after pretreatment with MβCD (318.4 ± 18.3 $\mu A/cm^2$, N=10, $P>0.05$). This suggests that AC is not involved in adenosine receptor activation of I_{sc} after cholesterol depletion. We also did not see a contribution of cAMP-dependent K^+ channel activity in the enhanced NECA response after cholesterol removal, and this suggests that the response is not due to the classical A_2 signaling pathway.

DISCUSSION

Here we present evidence that membrane cholesterol mediates adenosine receptor activation in colonic epithelium. Adenosine activated basal I_{sc} when applied to the apical or basolateral membrane of colonic epithelium, suggesting the presence of adenosine receptors in both membranes. Consistent with our results, adenosine receptor activation in both membranes has previously been shown in colonic epithelial cells (2). Barrett et al. also showed a greater activation of basolateral receptor-stimulated I_{sc}, compared to apical receptor stimulation, which we also observed with both adenosine and NECA responses.

Many studies have shown that MβCD significantly reduces the amount of cell membrane cholesterol (14;30), and we also observed this in colonic epithelial cells. In our studies, depletion of basolateral membrane cholesterol caused an increased response to basolaterally applied adenosine receptor agonists. This effect was not due to a decrease in nucleoside transporter activity, which would decrease the amount of adenosine uptake and increase the extracellular adenosine concentration. Exclusion of basolateral nucleoside transporter activity was shown by: 1) the use of NBTI and dipyridamole to inhibit the ENT1, and 2) the response to NECA (an adenosine analog that is not permeable through nucleoside transporters) after basolateral cholesterol removal, which was nearly identical to the adenosine response. Basal responses to NBTI and dipyridamole were relatively small, suggesting a low basal activity of ENTs. However, local increases in adenosine concentration are not easily measured in the Ussing chamber system since the continual perfusion would diffuse high local concentrations. Since we did not see a significant response to basal ENT inhibition, we could conclude that alterations in membrane cholesterol were unlikely to inhibit ENTs and cause the enhanced activation in I_{sc} that we observed. These results suggested that the effect is adenosine receptor-dependent.

Since the response to the A_{2A} receptor selective agonist CGS-21680 mimicked the effects of both adenosine and NECA, it appears that the basolateral A_{2A} receptor is mediating the increased response to adenosine agonists after basolateral cholesterol depletion. Furthermore, A_1 and A_3 receptor involvement was eliminated using the selective receptor agonist CPA and antagonist MRS-1523, respectively. Upon ligand binding the A_{2A} receptor activates the G_s protein, with subsequent AC activation, increased cAMP levels, and PKA activation. However, we observed no difference in forskolin response after basolateral cholesterol removal and this suggests that AC signaling is not affected. Furthermore, cAMP-dependent K^+ channels were not involved in the enhanced NECA response after cholesterol depletion.

There is evidence that the A_1 and A_{2B} receptors can be associated with caveolar membrane microdomains (21;31), and so adenosine receptors might functionally depend on membrane microdomains, or lipid rafts. Lipid rafts are characterized by enrichment in cholesterol and sphingolipids and certain membrane proteins. To determine if the effect we saw was dependent upon cholesterol- and sphingolipid-rich microdomains, we studied lipid raft disruption by three means, other than cholesterol depletion with MβCD: 1) using filipin, a cholesterol-binding reagent, 2) depleting sphingomyelin using sphingomyelinase, or 3) using colonic epithelium from mice lacking caveolin-1 and caveolae. MβCD has been shown to disrupt caveolae (16), which are characterized by the presence of caveolin-1 and are considered a subset of lipid raft. None of these three approaches to alter lipid raft integrity mimicked the effect of MβCD on adenosine receptor activation. Since A_1 receptors are caveolae-associated, this was further confirmation that our effect was not due to A_1 receptor activation. We also showed that cholesterol repletion after MβCD treatment caused a reversal of the NECA response to levels comparable to those without MβCD treatment. This showed that the enhanced NECA response was due specifically to alterations in membrane cholesterol, and that the effect was reversible.

The presence of cholesterol adds rigidity to the cell membrane and restricts the lateral motion of molecules, otherwise known as fluidity. Decreasing membrane cholesterol content has been shown to correspond with increases in membrane fluidity (5;14). Our studies of cell membrane fluidity showed that only MβCD, not filipin or sphingomyelinase, increased membrane fluidity. Our results confirm those of another study that saw a significant increase in membrane fluidity with MβCD treatment, but not with sphingomyelinase treatment (14). Therefore, we could conclude that the effect we were seeing was raft-independent, and exclusively related to cholesterol content and its effects on membrane fluidity.

Changes in the level of membrane cholesterol are known to affect membrane transport (30;32) as well as G protein-coupled receptor (GPCR) function (14;27). Although it is known that protein-lipid interactions are important for membrane function, these interactions are not well understood, and the molecular mechanisms of altering membrane cholesterol on membrane protein function are unknown. When cholesterol is depleted and membrane fluidity is increased, is it a simple matter of increased lateral diffusion, and therefore activation, of components of the A_{2A} receptor signaling pathway? Or does protein conformational change play a role to cause greater activity? Further studies are needed to answer these questions. Membrane cholesterol homeostasis is a highly regulated process (6), and it is plausible that epithelial cells could regulate adenosine signaling by controlling the amount of cholesterol in the membrane.

Cell membrane cholesterol depletion can have an inhibitory (14;17) or stimulatory (3) effect on a particular GPCR. It may be that membrane cholesterol depletion results in a reduction in ligand binding to certain GPCRs (14). In some cases altering membrane cholesterol may not have any effect on the activity of a membrane protein, such as sucrase, maltase and lactase activities in intestinal epithelium (5). In our case, we observed an increased response to GPCR stimulation after membrane cholesterol depletion. Although the regulatory role of cholesterol on a specific protein certainly depends on many factors, cholesterol-protein interactions could be important for some GPCRs (28). Recent evidence from Canal et al. has shown that A_{2A} receptors exist as functional homodimers, not monomers, at the cell surface, and also that cholesterol depletion does not affect homodimerization, as assessed by bioluminescence resonance energy transfer (7). This complements our data in which functional A_{2A} receptor activity still appears after cholesterol depletion.

Our results suggest that basolateral IK channels mediate K^+ secretion induced by cholesterol-dependent adenosine receptor activation. Altering membrane fluidity by manipulating membrane cholesterol content has been shown to affect several types of K^+ channels, including inwardly rectifying K^+ channels (30) and large conductance Ca^{2+} regulated K^+ channels (4). However, it is unlikely that IK channel activity is directly enhanced after cholesterol depletion since our previous studies showed that the IK channel was not involved in I_{sc} activation by membrane cholesterol depletion (20).

Several lines of evidence show the existence of multimolecular signaling complexes containing GPCRs and ion channels (9;22;26). This would be an efficient way to organize cellular signaling since activation of one type of GPCR would be selective for certain downstream targets. Perhaps the A_{2A} receptor also resides in an analogous signaling complex with IK channels. Intuitively, it makes sense that this signaling complex would be under a state of relative inhibition, given that the molecules are in close proximity of each other. By depleting cell membrane cholesterol, disinhibition of the normally quiet multimolecular complex would result in overreactivity upon ligand binding.

In these studies, we show that altering the cholesterol content of epithelial cell membranes affects adenosine-mediated ion transport. Basolateral adenosine receptor stimulation caused a greater secretory response after cholesterol depletion, and this effect was reversible upon cholesterol repletion. IK channels mediated the response to NECA and perhaps they reside in a basolateral multimolecular complex with A_{2A} receptors to specifically regulate secretion. Using a variety of raft-disrupting methods we observed that this effect was not due to the presence of membrane lipid microdomains, instead alteration of membrane cholesterol content and the concurrent change in membrane fluidity were responsible for these effects. Therefore, cellular regulation of membrane cholesterol content may control epithelial secretion.

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CHAPTER 5

Discussion

Epithelia have many roles, including protection of the tissues lying below, secretion of substances, and transcellular transport of molecules. Colonic epithelium is under the influence of many extracellular regulatory factors, which can originate from nerve, immune, and circulating cells. These mediators can affect ionic conduction across epithelial cells. In addition, intrinsic cellular mechanisms can regulate epithelial ion transport. The holistic function of the colonic epithelium is a result of the contribution from all regulatory mechanisms.

This thesis shows experimental evidence for several novel molecular mechanisms regulating epithelial secretory processes in the colon. We have further characterized neuroendocrine mechanisms that regulate colonic secretion (Chapters 2 and 4). We also looked at cell membrane composition and how it is important for regulating colonic epithelial ion transport (Chapters 3 and 4). Our studies emphasize that altering basolateral K^+ channel activity controls colonic secretion (Chapters 2, 3, and 4). The major findings from these studies are that:

- α_2-adrenergic receptor (α_2AR) stimulation inhibits colonic epithelial Cl^- secretion by inhibition of basolateral K_{ATP} channels
- basolateral BK channels in colonic epithelial cells are regulated by basolateral membrane microdomains
- adenosine receptor stimulation activates anion secretion in a membrane cholesterol-dependent manner

Cellular mechanisms of neural regulation of colonic epithelial ion transport

Neural inputs represent a major mechanism for regulating colonic secretion. The colonic epithelium receives innervation from the ENS (intrinsic nerves), as well as the ANS (extrinsic nerves), and these inputs contribute to inhibiting or activating epithelial secretory processes. Undergraduate-level physiology courses teach that sympathetic input inhibits intestinal secretion (via circulating catecholamines in the flight-or-fight response), and colonic epithelial α-adrenergic receptor stimulation corresponds to this generalization. The colon epithelium has a net absorptive function under normal conditions, and the pro-absorptive properties of sympathetic tone could help to maintain the balance of transepithelial ion transport. Although it well known that stimulation of colonic epithelial α_2ARs induces absorption (22), the cellular mechanisms involved in this response have not been fully studied. Chapter 2 elucidated that the absorptive action of α_2AR stimulation is due to inhibition of basolateral K_{ATP} channels. This represents a novel mechanism of K_{ATP} channel regulation by α_2ARs, and therefore neural regulation. Furthermore, this effect was shown to involve $G_{i/o}$ proteins, but not cAMP or Ca^{2+} signaling.

I_{sc} inhibition was due to a decrease in net Cl^- secretion, as assessed by pharmacological and $^{36}Cl^-$ studies. The fact that there was an increase in apical-to-basolateral movement of Cl^- suggests that basolateral Cl^- channels may be involved in the response to α_2AR agonists. It is not surprising that Cl^- secretion contributes to basal I_{sc}, however we found there was a bidirectional transepithelial movement of Cl^- so that Cl^- absorption was also present under basal conditions. This backflux of Cl^- must involve basolateral Cl^- channels. Basolateral Cl^- channels are present in mouse colon epithelial cells (32), and we have found an increase in basal I_{sc} in response to basolateral Cl^- channel inhibition using the inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS, 1 mM, unpublished observations). This suggests that basolateral Cl^- channels are constitutively open under normal conditions, and would allow for a backflux of Cl^- movement. The molecular identity of basolateral Cl^- channels in colon epithelium is largely unknown, however ClC-2 channels may be located in the basolateral membrane (8) where they could contribute to colonic Cl^- absorption. Further studies are needed to clarify a role, if any, for basolateral Cl^- channels in the response to α_2AR agonists.

Our studies showed that basolateral K_{ATP} channels are inhibited in response to α_2AR agonists; however, NKCC and the Na^+/K^+-ATPase were not inhibited. α_2AR agonists inhibit K_{ATP} channels, and the closing of these channels drives a reduction in colonic Cl^- secretion. These studies also showed mRNA expression of K_{ATP} channels in mouse colon epithelium. K_{ATP} channels are unique K^+ channels in that they have an octomeric structure, as opposed to the classical K^+ channel structure containing 4 subunits. They are made up of 4 Kir6.x K^+-conducting subunits, and these are surrounding by 4 sulfonylurea receptor (SUR) regulatory subunits (1). From evaluation of K_{ATP} channel subunit mRNA expression as well as pharmacological studies, it was inferred that mouse colonic epithelial cells contain K_{ATP} channels similar to those in vascular smooth muscle cells, which are made up of Kir6.1/SUR2B subunits or Kir6.2/SUR2B subunits. These channels are tonically active, since basal I_{sc} was inhibited by K_{ATP} channel blockers. K_{ATP} channels are inhibited by ATP (37), and activated by phosphatidylinositol phosphates (4;43). The balance in mouse colon epithelial cells must lean towards open K_{ATP} channels under normal conditions, since we found that K_{ATP} channels were basally active. Basolateral K_{ATP} channels may therefore contribute to basal Cl^- secretion for colonic hydration.

α_2ARs can couple to $G_{i/o}$ proteins (11), which are inhibited by pertussis toxin, as well as G_s proteins (14). We saw that α_2AR agonists inhibited I_{sc} in a pertussis toxin-sensitive manner. However, neither cAMP nor Ca^{2+} mediated the response to α_2AR agonists. This suggests that other intracellular signaling mechanisms are responsible for transducing the signal from α_2ARs to K_{ATP} channels. $G_{i/o}$ proteins have been shown to directly activate K_{ATP} channels (23), and perhaps this occurs in colonic epithelium. Further studies are needed to clarify the intracellular signaling mechanisms regulating the response to α_2AR activation.

α_2AR agonists inhibited I_{sc} to a greater degree in proximal than distal colon epithelium. The responses to α_2AR subtype-specific inhibitors suggested that the α_{2A} subtype of ARs are present in mouse colon epithelium, in accordance with human colonic epithelial cells (48). Why do α_2AR agonists have more potent effects in the proximal colon? Likely, α_2AR expression is higher in the proximal mouse colon, as has been shown in human intestine (48). Alternatively, or additionally, K_{ATP} channels may show more abundant expression in the proximal colon. If basolateral Cl^- channels are involved in the response to α_2AR agonists, then maybe these channels also show higher expression in the proximal colon. Future qualitative studies could determine the expression pattern for these channels along the length of the colon.

Cell membrane composition regulates colonic epithelial ion transport

Chapter 3 studied cell membrane regulation of epithelial ion channels. We provide evidence that the cell membrane lipid environment plays a role in regulating colonic epithelial ion transport. These studies highlighted the polarity of epithelial membrane function, since the effect of altering lipid composition in the apical membrane was different from the same experiments performed on the basolateral membrane. In these studies, the two lipids of concern were cholesterol and sphingomyelin. Lipid rafts are enriched in both cholesterol and sphingomyelin, and rafts have been assigned important roles in regulating certain membrane proteins, including ion channels. Our studies focused on these membrane microdomains in colonic epithelial cells.

Our studies of membrane lipid composition and its regulation of colonic epithelial ion transport showed that basolateral BK channel activity depends on basolateral lipid raft integrity. We used three different methods to disrupt rafts: methyl-β-cyclodextrin (cholesterol depletion), filipin (cholesterol binding), and sphingomyelinase (sphingomyelin depletion). Basolateral raft disruption was shown to activate transepithelial CFTR-mediated anion secretion, and this was due to activation of basolateral BK channels. As previously mentioned, basolateral K^+ channel activation is one mechanism for driving anion secretion, and this is an example of how the coordinated activity of ion transporters contributes to net ion transport. We also looked at other possible basolateral membrane targets such as NKCC and the Na^+/K^+-ATPase, which has been shown to be raft associated (28); however, neither of these transporters was involved in this response. Addition of exogenous ceramide, a product of sphingomyelin hydrolysis, did not mimic the effect of SMase. Ceramide is known to affect certain ion channels (5), however our data suggests that ceramide production is not involved in the raft-dependent response we observed.

Does this effect occur in other types of epithelial cells? Similar experiments in our lab using a human airway epithelial cell line (Calu-3) showed that basolateral raft disruption with MβCD did not activate I_{sc} (unpublished observations). Therefore, BK channel regulation by rafts does not seem to be a universal regulation mechanism in all epithelial cells.

Immunoblotting experiments showed the protein expression of BK channels in mouse colonic epithelium. In addition, since BK channel inhibition did not have an effect on basal I_{sc}, these channels are tonically inactive, unlike K_{ATP} channels. From the sucrose gradient density fractionation studies, the BK channel was found predominantly in the high-density pellet fraction. Although the BK channel did not localize to light density fractions, where raft proteins typically localize, this does not mean that the BK channel is not raft associated. Cytoskeletal proteins have been shown to isolate in high-density detergent-insoluble domains (36), and many cytoskeletal proteins are found associated with rafts (26;44;49). We hypothesize that BK channels reside in high-density cytoskeletal-associated rafts. Caveolar BK channels have been shown to be associated with, and regulated by, the actin cytoskeleton (7). Continuation of this project would benefit from studies looking at colocalization of colonic epithelial BK channels with cytoskeletal components, for example using immunocytochemistry or coimmunoprecipitation experiments. It is not understood how membrane proteins associate with lipid rafts, and cytoskeletal attachment represents one mechanism for raft protein association. Ankyrin, a protein that binds membrane proteins to the cytoskeletal network, appears to be located near the basolateral pole of intestinal epithelial cells (21). In the polarized epithelial cell, the cytoskeletal network underlying the basolateral membrane is distinct from that under the apical membrane. For example, the microtubule network in Caco-2 cells is dense under the apical membrane and sparser near the basolateral domain (16). Cytoskeletal organization and spatial location could be part of the reason that apical and basolateral rafts differ. Indeed, our studies showed a different effect on transepithelial transport from raft disruption at the apical or basolateral membrane.

Caveolar BK channels associate with caveolin-1 in vascular endothelial cells (50) and myometrial smooth muscle cells (7), and in both of these cells cholesterol depletion activated BK channels, which corresponds to our data. However, BK channel association with caveolin-1 might depend on cell type, since the two proteins colocalized in esophageal skeletal muscle bundles, but not in intestinal smooth muscle cells (10). Our studies showed that caveolin-1 protein is expressed in mouse colon epithelial cells. Caveolin-1 is localized in light-density fractions, as well as the pellet fraction, and raft disruption altered caveolin-1 localization. Although we did not see colocalization of BK channels and caveolin-1 in light-density fractions, it is possible that the caveolin-1 in the pellet fraction is associated with BK channels in colonic epithelial cells.

Several types of ion channels are raft-associated and rafts represent a membrane-intrinsic mechanism for channel regulation. Numerous signaling molecules also localize to rafts. BK channels are regulated by and associated with PKA (47), and G proteins directly modulate BK channel activity (25). Membrane microdomains represent a novel mechanism for BK channel regulation, which includes channel association with the cytoskeleton and other proteins to maintain the channel's raft localization. In support of this organizational model for ion channels, Trp Ca^{2+} channels were shown to associate with caveolin-1 and to functionally depend on the actin cytoskeleton (29). Coimmunoprecipitation experiments did not show a direct interaction between the Trp channels and actin (29); however, other proteins may cross link channels with the cytoskeleton. Another study shows evidence that K_{ATP} channels are compartmentalized with AC by their association with caveolin-1 (42). Signaling and regulatory molecules located within close vicinity of BK channels would create an organized package of molecules that could regulate BK channel function. Support for this idea comes from studies showing that BK channels are found in multimolecular signaling complexes in smooth muscle cells (27). Further studies are needed to clarify if BK channels are associated with caveolin-1 and with cytoskeletal proteins in colon epithelial cells.

Cell membrane composition affects endocrine regulation of colonic secretion

Physiologically, adenosine is an important regulator of colonic epithelial secretion under basal conditions, with local autocrine and paracrine production. Under pathological conditions, adenosine can be produced in large quantities by non-epithelial cells with consequences on colonic secretion. Although it is well known that adenosine stimulates epithelial secretion, the cellular mechanisms of adenosine action are not clearly defined. The studies from Chapter 4 define a mechanism of regulation for adenosine signaling in colon epithelial cells, where cell membrane composition influences adenosine-mediated secretion.

Our studies on the regulation of adenosine receptor-mediated ion transport present novel and interesting results. We showed that altering membrane cholesterol content affected the response to certain adenosine receptor agonists, specifically A_{2A} agonists. The influence of cell membrane lipids on membrane proteins is unquestionable, however the specificities of lipid regulation of protein function are not well known.

The hydrophobic regions of membrane proteins are associated with lipids in the cell membrane, and it is generally accepted that these proteins require a specific lipid environment to function properly. There are different schools of thought on what kind of role the lipid-protein interaction has. Do proteins "choose" the lipids that they will associate with? Or do the lipids determine the proteins they associate with? Because of the great diversity of lipid species in the cell membrane, it would seem that there are quite specific lipid-protein interactions that are not randomly determined. One theory for explaining how some proteins are targeted to lipid rafts describes "lipid shells" that encase the proteins and target them to rafts (2). This model supports that lipids determine the protein's location.

Cholesterol is enriched in the cell membrane, compared to intracellular membranes. Certain GPCRs have been shown to rely on membrane cholesterol for proper functioning, and cholesterol depletion may inhibit (17;19;20) or stimulate (3;6) GPCRs. Although cholesterol may have indirect effects on membrane proteins, it also can have direct effects. Studies with optical isomers of cholesterol are a thorough method for showing cholesterol-specific actions (41). Cholesterol may directly bind and regulate membrane proteins, such as caveolin-1 (34), or it may regulate them indirectly. Membrane cholesterol depletion was shown to reduce ligand binding for certain GPCRs (17;20;38).

Our studies showed that manipulation of basolateral membrane cholesterol levels, but not another cell membrane lipid (sphingomyelin), affected adenosine-mediated secretion in the colon. This effect was not due to disturbances in lipid raft integrity, since other raft-disrupting treatments did not affect the response to basolateral adenosine receptor stimulation. We also showed that the effect was associated with cholesterol's effects on membrane fluidity. This suggests that the effect of cholesterol on the physical parameters of the cell membrane may be mediating the response.

With respect to epithelial polarity, cholesterol is found in larger amounts in the basolateral compared to the apical membrane of rat intestinal epithelial cells (9). The basolateral membrane of MDCK epithelial cells showed a faster rate of cholesterol efflux than the apical membrane (39), and perhaps this also is a factor in intestinal epithelial cells. Physiological cholesterol efflux occurs at the basolateral membrane of colonic epithelial cells, where HDL mediates reverse cholesterol transport via the circulation. This process contributes to cellular cholesterol homeostasis, and from our data we hypothesize that cholesterol efflux also regulates adenosine-mediated colonic secretion.

RT-PCR studies showed ENT1, but not ENT2, mRNA expression in mouse colonic epithelial cells. Studies in T84 cells show that ENT activity is restricted to the basolateral membrane (33;46). On the other hand, CNT uptake occurs in the apical membrane of small intestinal epithelium (40), and was completely absent in colon epithelial cells (40;51). Although we showed ENT1 expression mouse colon epithelium, pharmacological studies excluded nucleoside transporter involvement in the enhanced response to adenosine receptor agonists that we observed.

Our studies suggested that the basolateral IK channel is involved in the enhanced secretory response that we observed. IK channel activity is likely not directly affected by cell membrane cholesterol, since results from Chapter 3 showed that the IK channel was not involved in the response to MβCD. The presence of basolateral IK channels has been observed in rat colon epithelial cells (15), and these channels have been shown to play a role in colonic secretion (24). In mouse colon epithelium, basolateral IK channels are not basally active since IK channel inhibition did not affect basal I_{sc}. However, these channels may be activated in response to adenosine. Studies in airway epithelial cells suggest that adenosine activates IK channels (45), and perhaps this also occurs in colonic epithelial cells.

One explanation of the results from these studies is that the basolateral membrane in colonic epithelial cells contains molecular complexes bringing A_{2A} receptors and IK channels in close proximity. Whole signaling complexes containing GPCRs and their effectors have been identified. For example, the β_2-adrenergic receptor was found associated with G_s, AC, PKA, phosphatase PP2A, and an L-type Ca^{2+} channel (12). Another study using airway epithelial cells revealed a molecular complex containing the β_2-adrenergic receptor, AC, EBP-50, and CFTR (35). These multimolecular signaling complexes would create an environment for efficient and selective signal transduction, from GPCRs to their nearby effectors.

The studies in this thesis answer several questions, but also bring to light many new questions. New methods are continually being developed and can help to more thoroughly answer some of these questions. For example, membrane cholesterol studies would benefit from using *in vivo* treatments to alter cholesterol content. It was shown that feeding mice *n*-3 PUFAs caused a decrease in the amount of cell membrane cholesterol by ~45% in colonic epithelial cells (30). This technique could bridge this research to clinical applications, where diet could alter cell membrane cholesterol content. Recently mice were produced that are deficient in desmosterol reductase, an enzyme involved in cholesterol biosynthesis, and these mice have greatly decreased plasma cholesterol levels (52). It is possible that these mice also have decreased level of cell membrane cholesterol, and therefore it would be interesting to use them for continuation of our studies on colonic epithelial ion transport.

Experimental model

The most commonly used human cell lines for studies of colonic epithelial ion transport are T84, HT-29, and Caco-2. Although cell lines afford many benefits for studying the cellular mechanisms controlling ion transport, they also have some drawbacks. One major issue is that their ion channel expression profile can differ dramatically from native tissue. It is highly unlikely that these cell lines behave like native cells in colonic epithelial tissue. The colon epithelial cell lines that are currently used most resemble crypt base cells (18). However, whole tissue secretory responses involve a heterogenous population of epithelial cells situated along the crypt base to the surface, which have varying ion channel profiles (e.g. CFTR, ENaC).

One benefit of tissue experimental models is that native tissue responses are studied. The colonic epithelial tissue model we used more closely mimics the *in vivo* situation than colonic epithelial cell lines grown in culture. In our experiments, epithelial tissue is freshly isolated and conditions are imitated to maintain the tissue in its native state as closely as possible. Although tissue models are closer to the *in vivo* situation than cell lines, their more complex structure also can be a drawback in the experimental situation. As with all experimental models, the potential limitations need to be acknowledged. Rarely is a tissue sample a homogenous group of cells like cell lines are. Aside from underlying muscle cells, several other cells are found in the submucosa including neurons, mast cells, intraepithelial lymphocytes, and fibroblasts. It is not known what contribution these cells have in altering epithelial function. In the colonic epithelial tissue used for our Ussing chamber studies, the muscular layers are carefully dissected from the epithelium. Once the tissue is mounted in the Ussing chamber system, continuous perfusion with $95\%O_2/5\%CO_2$ allows a gentle washing of the epithelial membranes, reducing the stray cells associated with the tissue in the submucosa.

Intestinal epithelial cells are extremely difficult to grow as primary cultures because they are fragile cells. Isolation of these cells is tedious since they die within hours of isolation. Performing experiments (patch-clamp studies, intracellular Ca^{2+} measurements, etc.) on isolated cells is not easy, and has limited some experiments that we could have done. Whitehead et al. have created a cell line of mouse colon epithelial cells, called young adult mouse colon (YAMC) cells (53). Perhaps these cells are more robust and could better handle cellular experiments requiring isolated cells. If so, these cells would be useful for continuing studies on cellular mechanisms regulating colonic epithelial ion transport.

Common animal tissue models for colonic transepithelial ion transport studies have traditionally been the rat, rabbit, guinea pig, and mouse. For our studies we have used mouse colonic tissue. Although species differences are evident, mice are decent animal models for observing gastrointestinal function with applicability to the human condition. For example, CF mouse intestinal pathology, namely gastrointestinal obstruction, is similar to that seen in CF patients. Due to the similarities of gastrointestinal dysfunction in both CF patients and mice, this suggests that intestinal function in both mice and humans relies heavily on the presence of CFTR for proper secretory function.

Conclusion

The studies in this thesis show that basolateral K^+ channels play an important role in transepithelial colonic secretion. Many studies have shown the importance of basolateral K^+ conductance in Cl^- secretion (13;31). In fact modulation of basolateral K^+ channels may be effective for treatment of dysfunctional intestinal secretion. At least in theory, basolateral K^+ channel activators may help alleviate hyposecretion, whereas inhibitors may help cases of secretory diarrhea.

Taken together, our results show several different novel mechanisms of regulating basolateral K^+ channel activity and overall colonic secretion. These findings further our understanding of the complex mechanisms regulating colonic epithelial ion transport, such as membrane composition and neuroendocrine factors. The information from these studies could contribute to the development of specific treatments for conditions of colonic hypersecretion or hyposecretion, and highlights the need for further study in this area.

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